

# CRANFIELD UNIVERSITY

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**STAVROPOULOU ANDRIANA**

**ESSENTIAL OILS FROM DITTANY OF CRETE FOR THE  
CONTROL OF *BOTRYTIS CINEREA* ON TOMATO, EGGPLANT  
AND PEPPER FRUITS**

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CONTROL OF *BOTRYTIS CINEREA* ON TOMATO, EGGPLANT  
AND PEPPER FRUITS**

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## Abstract

The degree of postharvest losses of fruit and vegetables due to attack by microorganisms worldwide is significant. In addition, the use of chemicals increases consumer concerns regarding food safety. Recent studies indicate that exploitation of natural compounds such as essential oils (EOs) may provide an alternative and friendlier way for the preservation of fresh produce. In this research project the efficacy of *Origanum dictamnus* L. essential oils (EOs) (a medicinal-aromatic plant of Crete) for the control of *B. cinerea*, a common postharvest pathogen of three economically important Cretan vegetables, tomato, pepper and eggplant was examined. Pathogen development in culture medium or on fruits was evaluated after treatment with dittany EO (0, 50, 100, 250 ppm) in vitro and in situ when stored at 12°C and 95% RH during or following exposure to EO volatiles. The impacts of short term exposure of *B. cinerea* to volatiles on growth and sporulation were also studied. Fungal development in vitro was completely inhibited by the continued application of 100 or 250 ppm of EO volatiles while inhibitory effects were also marked by lower EO concentration and short time of exposure. In inoculated fruits the 50 ppm of EO application resulted in suppressed disease development by reduced lesion growth and fungal sporulation, where increasing EO concentration led to greater effects. Pre-exposure of fruits to volatiles before fungal inoculation revealed reduced lesion growth, indicating that dittany EO probably caused induced resistance of fruits against the pathogen. Moreover, EO application did not affect quality-related characteristics of fruits while skin lightness and pulp lightness of eggplant fruits was improved under the presence of dittany volatiles. Overall, the results suggest that dittany EO volatiles may be considered as an alternative food preservative treatment, significantly reducing or eliminating *B. cinerea* infection during fruit storage and perhaps also in transit.

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## **CHAPTER 1**

### **Introduction - Literature Review**

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## **1.1 Definition of the problem**

The degree of postharvest losses of fruits and vegetables has been of growing concern in the food industry in recent years. Postharvest losses of fresh produce represent a critical component affecting global food losses (FAO, 2011). Several studies point out that in order to meet the increasing food demand in the future, postharvest losses must be reduced without increasing the burden on the natural environment ensuring future global food security (Kader, 2005; Hodges et al., 2011).

Losses in perishable fresh produce such as fruits and vegetables can occur along the postharvest chain from harvesting, through handling, storage, processing and marketing to final delivery to the consumer (FAO, 1989). These losses can be quantitative or qualitative making products unfit for human consumption. Postharvest diseases, particularly due to fungal infection, represent a significant factor that causes the major losses of fresh produce by reducing their quantity, quality or both. Perishable fruits and vegetables have a high water and nutritional content which makes them a rich habitat for microorganisms. Perishable fruit are stored at a high relative humidity to conserve quality. Thus, during short and medium term storage they are highly susceptible to attack by pathogenic microorganisms, especially where skin damage has occurred (Agrios, 2005; Boyraz and Özcan, 2006; Pitt and Hocking, 2009). The degree of postharvest losses of fruits and vegetables due to attack by microorganisms worldwide is estimated to be between 10 and 30% of the total crop yield while in developing countries may be significantly higher (Agrios, 2005).

Chemical treatments, cold storage and modified atmosphere storage techniques are the primary means for controlling postharvest decay of fruits and vegetables (Pramila and

Dubey, 2004; Agrios, 2005). The continuous use of fungicides over the last few decades has resulted in concerns about (a) consumer concerns over the safety of foods containing synthetic chemicals, (b) environmental concerns over excessive use of chemicals and the resultant residues, and (c) the development of fungicide resistance in economically important fungal pathogens (Brent et al., 1998; Pramila and Dubey, 2004; Tzortzakis, 2009b,).

Obstacles and constraints arising over the use of chemicals generated the need for alternatives to fungicides for the control of postharvest diseases of fruits and vegetables. Thus, the use of chemicals for the control of postharvest decay is restricted in most countries due to potentially carcinogenic residues and the withdrawal of a number of fungicides for postharvest application by the EU. This has become a major driver for the development of alternative disease control methods to be found (Wilson et al., 1991; Tzortzakis, 2009b). Alternative strategies to control postharvest disease that have been investigated the last 20 years include the induction of resistance in fruit, the use of plant or animal products with fungicidal activity and the application of antagonistic microorganisms (Spadaro and Gullino, 2004). Although there has been significant progress in research, practical alternative preservation methods have not often been effective or consistent enough, while a limited range of commercially biocontrol products are available (Droby et al., 2009). One possible alternative postharvest disease control method, is the utilisation of natural essential oils (EOs) from plants as antimicrobial agents for the preservation of fruit and vegetables (Serrano et al., 2005; Tzortzakis and Economakis, 2007; Tzortzakis, 2009a; Eva Arrebola et al., 2010; Sharif et al., 2010; Soylu et al., 2010; Thierry et al., 2010; Tzortzakis, 2010; Ugo De et al., 2010; Combrinck et al., 2011; Jun et al., 2011).

Tomatoes, eggplants and peppers are wide consumed vegetables worldwide due to their nutritional value. These products as part of the Mediterranean diet are widely grown in Crete and generally in Greece (Table 1.1). Part of these commodities production goes to the fresh market, another part is standardized and packaged for distribution domestically and a significant amount is exported (Table 1.2). These perishable products are highly susceptible to attack by fungal pathogens, especially *Botrytis cinerea*, both pre- and post-harvest. *B. cinerea* Pers.: Fr. is a ubiquitous fungus which causes grey mould rot and reduces product quality and yield (Agrios, 2005; Elad et al., 2007; Pitt and Hocking, 2009). Grey mould is responsible for the major economic losses of production of these three commodities.

**Table 1.1:** Official data of the statistics division of FAO (FAOSTAT 2014) for the harvested area (ha) in Greece of tomatoes, eggplants and peppers the years 2009-2011 <http://faostat3.fao.org/faostat-gateway/go/to/download/Q/QC/E>.

Greece	Area harvested (ha)		
	Year		
Item	2009	2010	2011
Tomatoes	32350.00	24200.00	19800.00
Eggplants	3098.00	2600.00	2500.00
Peppers	3902.00	3900.00	3500.00

Taking into account the above, research on natural compounds which are ecologically safe and consumer-friendly to control key postharvest diseases and maintain fresh produce quality is an area which requires more research for potential practical use.

**Table 1.2:** Official data of the statistics division of FAO (FAOSTAT 2014) for the quantity (tonnes) of tomatoes, eggplants and peppers produced and exported the years 2009-2011 in Greece <http://faostat3.fao.org/faostat-gateway/go/to/download/T/TP/E>.

Greece	Quantity (tonnes)					
	Year					
	2009		2010		2011	
Item	Production	Export	Production	Export	Production	Export
Tomatoes	1561311.00	2639.00	1406200.00	7307.00	1169900.00	14470.00
Eggplants	67190.00	202.00	70900.00	387.00	78000.00	620.00
Peppers	120000.00	12319.00	141000.00	12624.00	130800.00	13107.00

## 1.2 Tomato, eggplant and pepper

### 1.2.1 Nutritional value

Eggplants (*Solanum melongena* L.), cherry tomatoes (*Solanum lycopersicum* L.) and peppers (*Capsicum annum* L.) belong to the family of *Solanaceae* and are widely consumed due their nutritional value. Tomato is considered a key protected horticultural crop providing about 25mg vitamin C per 100 g fresh weight and 47% of vitamin B, 33% of vitamin A, 0.3-0.6% minerals (calcium, potassium, magnesium, iron) of the daily dietary requirements of an average person from one medium tomato. According to research consumption of tomato on a regular basis can prevent deficiencies of vitamins and minerals (Rama and Narasimham, 2003). Eggplant, besides the vitamins and mineral content has been associated with cell membrane protection as it contains ‘nasunin’ a potent antioxidant and free radical scavenger (Matsubara et al., 2005). Pepper is considered a great source of different phytochemicals, including vitamin C, phenolic compounds, flavonoids, and carotenoids providing health-beneficial effects

(Zhuang et al., 2012). The nutritional quality will also be influenced by cultivar, maturity stage, agronomy and post-harvest treatment of the fruit (Rama and Narasimham, 2003; Zhuang et al., 2012).

### **1.2.2 Market quality indices**

Tomato quality is based on uniformity of shape and size together with freedom from growth or handling defects. Fruits with a well-formed shape, uniform colour without green shoulders and firm are desirable. Their appearance must be smooth with small blossom-end scar and stem-end scar with no visible sign of physical and physiological disorders like growth cracks and sun scald, mechanical injury such as wounds or cuts and no sign of decay (Cantwell and Suslow, 2009).

Eggplant quality indices are influenced by eggplant types (elongated or egg-shaped, purple, white or striped) and their marketing in different regions. Purple type of fruits is commercially more desirable. Their colouration is caused by anthocyanins in fruit skin (Concellón et al., 2007). Quality attributes for egg-shaped purple fruits include uniform egg to globular shape and bright dark purple skin colour as well as fresh green calyx and absence of seed or pulp browning. Fruits must be firm, free from growth or handling defects or any kind of decay (Cantwell and Suslow, 2009). Fruits under long term storage may suffer calyx discoloration and pulp browning caused by the oxidation of phenolic compounds (Massolo et al., 2011).

Peppers mature through three distinct stages: immature green, mature green and mature red. Mature green and mature red peppers are both horticultural mature and can be consumed fresh. Immature green fruits are poor in colour and flavour and unacceptable for marketing (Agblor and Waterer, 2001). Peppers must be uniform in shape and size

and must have uniform colour typical of the variety. Fruits which are firm and have no sign of defects such as cracks, decay, sunburn or flaccidity are successfully marketed (Cantwell).

### **1.2.3 Postharvest handling and storage**

Tomatoes are climacteric and highly perishable fruits. They are harvested at different stages of maturity depending on their use. After harvesting, fruits are cleaned and usually sanitized with sodium hypochlorite solutions (150 ppm) to eliminate spread of disease during storage. However, this method is not always effective with more tolerant fungal pathogens such as *Botrytis* or *Rhizopus* (MFCL, 2003b). The optimum storage temperature and relative humidity for tomato is 12 °C and 86–95% RH. Tomatoes are prone to chilling injury if stored below 10 °C for long periods of time. Chilling injury may result in rapid deterioration in quality and decay by allowing the entry of microbial pathogens. High relative humidity prevents water loss and extends postharvest quality of fruits. Controlled-atmospheres and packaging in polyethylene bags can also prolong the shelf-life of tomato (Rama and Narasimham, 2003).

Eggplant is a non-climacteric fruit (Massolo et al., 2011) harvested at a range of maturity stages depending on market demand. Fully or over- matured fruits are not acceptable because they are dull in appearance, with spongy flesh and the seeds turn brown. Fruits should be heavy in relation to their size when harvested and are graded as small, medium and large in size. Washing the fruits with sodium hypochlorite solutions (150 ppm) is a recommended treatment to minimise microbial infection. Eggplants do not have a long storage life even if they are not as perishable as tomatoes (MFCL, 2004). The optimum storage conditions for eggplants are 10-12.8 °C and 90-95% RH



for 2-3 weeks. Fruits are sensitive to temperatures  $<10^{\circ}\text{C}$  and chilling symptoms can occur in 6-8 days. High relative humidity prevents fruits from shrivelling. Controlled-atmosphere storage does not seem to extend the storage life of eggplants whilst pre-packaging of fruits in adequately ventilated polyethylene bags (100-gauge) can prolong shelf-life (Rama and Narasimham, 2003; Cantwell and Suslow, 2009).

Pepper is a non-climacteric fruit that must be cooled as soon as possible after harvest to reduce water loss which leads to flaccidity and shrivelling of fruit. Fruits have a large surface to weight ratio and water loss is mainly due to diffusion through the cuticle of the skin. Peppers are sensitive to chilling injury which limits storage temperature to  $>10^{\circ}\text{C}$  (Lerdthanangkul and Krochta, 1996; González-Aguilar et al., 1999). Symptoms of chilling injury include pitting, softening and decay (Cantwell). Storage at high RH of 90-95 % will maintain fruit firmness (MFCL, 2003a). Wax-coating of the fruits commonly reduces water loss and controlled atmosphere storage extends shelf-life of peppers (Rama and Narasimham, 2003).

#### **1.2.4 Most common postharvest diseases and recommended control methods**

Tomatoes, eggplants and peppers can be affected by both fungal and bacterial diseases postharvest. Economically, the fungal diseases may be more important, especially post-harvest. Chilling injury such as weak tissue and mechanical injury such as cuts and wounds but also natural openings around the calyx, stem end scar and blossom end scar can allow the entry of fungal pathogens (Pitt and Hocking, 2009).

Tomatoes may suffer severe postharvest losses due to decay or surface lesions commonly caused by *Alternaria* (Black Mould Rot), *Botrytis* (Gray Mould Rot), *Geotrichum* (Sour Rot) and *Rhizopus* (Hairy Rot) (Pitt and Hocking, 2009). Chemicals

such as captan, dithane, and thiram are recommended as fungicidal treatments applied postharvest on tomatoes along with good horticultural storage practices (Rama and Narasimham, 2003).

Eggplants' shelf-life is limited dramatically mainly by the development of fungal diseases postharvest. Grey mould, *Alternaria* rot, *Phomopsis* and *Rhizopus* rot represent the most common fungal disease of eggplant (Fallik and Grinberg, 1992). Eggplant is also susceptible to bacterial decay. Fungicidal wax emulsions can extend the shelf-life of eggplants by 30–40%. Immersions in hot water and a low concentration of a dithiocarbamate provides good disease control (Rama and Narasimham, 2003).

Peppers are highly susceptible to postharvest fungal infections mainly caused by *Botrytis cinerea* and *Alternaria alternata* which may result in considerable losses (Fallik and Grinberg, 1992; González-Aguilar et al., 1999). Hot water dips at 52-55°C for 2 minutes and application of registered antimicrobials such as O-phenylphenol at 98 g/L to control postharvest disease of peppers is recommended (Agblor and Waterer, 2001).

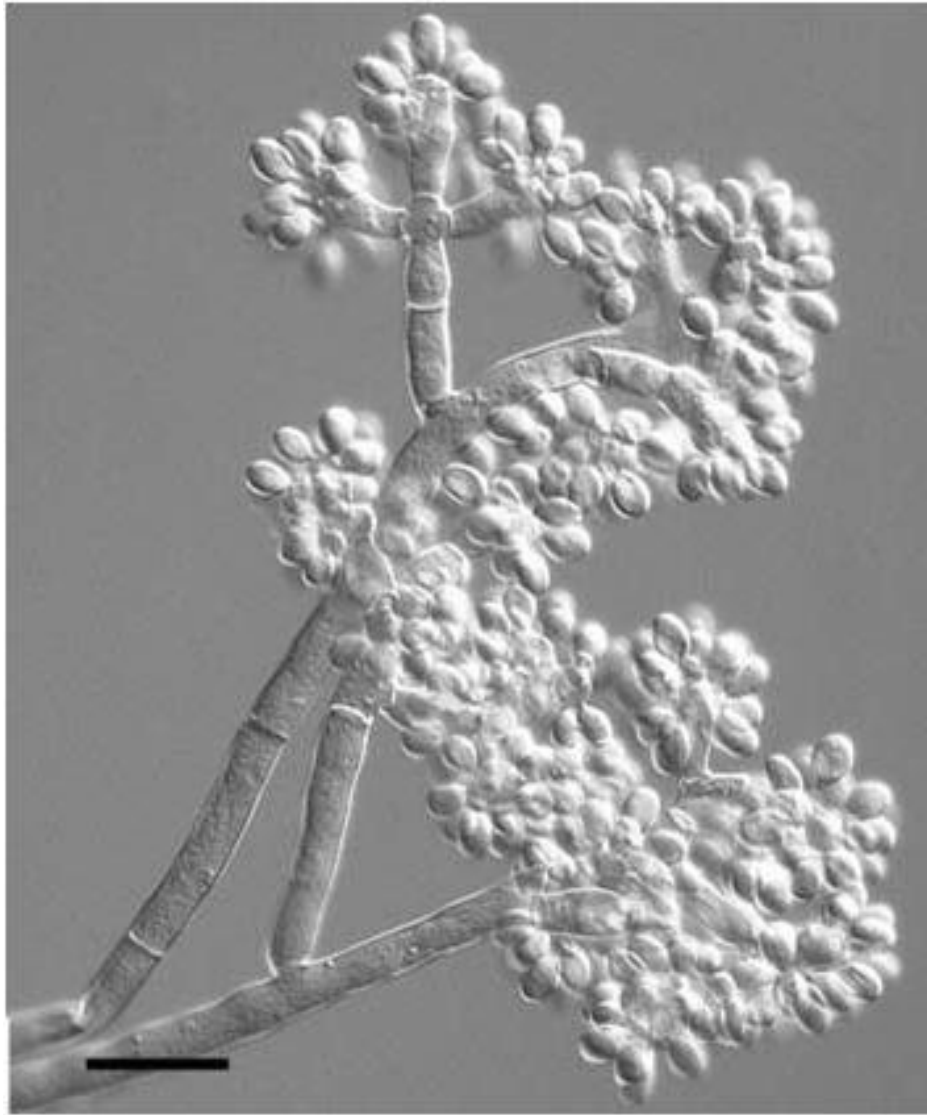
### **1.3 The pathogen (*Botrytis cinerea*)**

For more than 125 years, *B. cinerea* and other *Botrytis* species have been the focus of research on a wide range of crops for determining control measures from epidemiological and forecasting risk approaches to fungicides resistance to developing biocontrol strategies (Elad et al., 2007). Although other *Botrytis* species are specific to certain hosts, *B. cinerea* is a ubiquitous and polyphagous plant pathogen that causes

extensive damage pre- and postharvest to a wide range of economically important crops world-wide (Agrios, 2005; Elad et al., 2007).

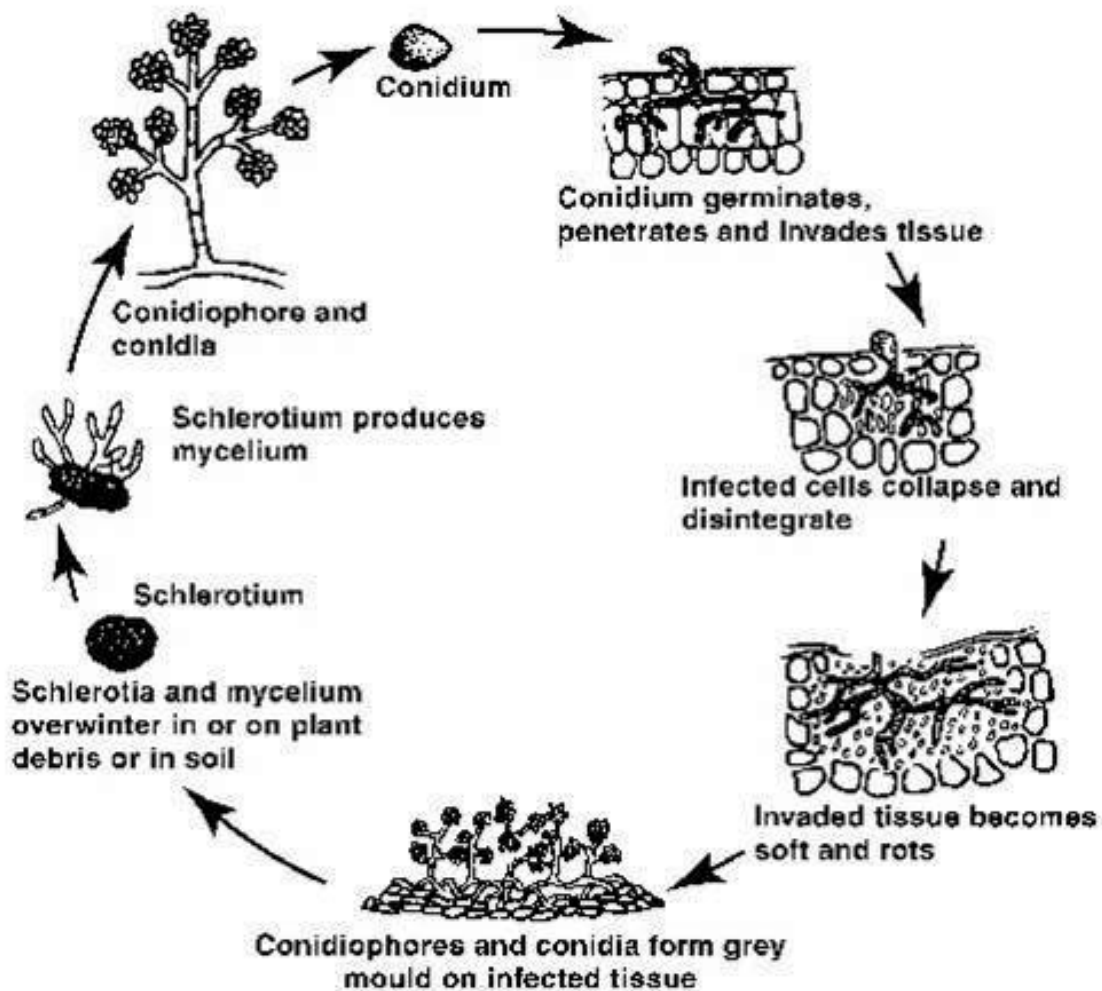
*B. cinerea* Pers.: Fr. is characterized as “imperfecti” (Barnes, 1979) (imperfect - asexual) and belongs to an artificial class of Deuteromycetes where fungi are grouped according to forms and not according to sexual characteristics because their perfect sexual state is rarely found (Barnes, 1979; Agrios, 2005). The anamorph or asexual state is *B. cinerea* and the teleomorph or sexual state in the life cycle of the fungus is *Botryotinia fuckeliana* (Elad et al., 2007).

The asexual state of the pathogen produces grey mycelium and branched conidiophores that have rounded apical cells bearing clusters of colourless or grey, one-celled, ovoid conidia. The conidiophores and clusters of conidia resemble a bunch of grapes-like cluster (Plate1.1). Conidia are released readily in humid weather and are carried by air currents (Agrios, 2005). The fungus frequently produces sclerotia when nutrients are limited. Sclerotia are black, hard structures and resistant to adverse environmental conditions (Markellou, 1999). *B. cinerea* occasionally produces the perfect stage *Botryotinia fuckeliana* in which ascospores are produced in an apothecium (Agrios, 2005).



**Plate 1.1:** Conidiophores and conidia of *B. cinerea* as it appears under an electron microscope, bar=25  $\mu$ m, (Pitt and Hocking, 2009).

*B. cinerea* causes grey mould or grey mould rot disease (Plate 1.2). The decay may start at the blossom or stem end of the fruit or at any wound. The decay appears as a well-defined water-soaked, then brownish area that penetrates deeply and advances rapidly into the tissue. In most hosts and under humid conditions a greyish or brownish-grey, granular, mould layer develops on the surface of decaying areas (Agrios, 2005) (Plate 1.3).



**Plate 1.2:** Life cycle of *B. cinerea* grey mould disease (OMAFRA, 2012).

The pathogen is capable of infecting hosts in more than one way and is able to penetrate both healthy, decaying and decayed tissue (Markellou, 1999). The conidia are able to remain latent on plant surface until a thin film of water is formed and then germinate (Markellou, 1999). The sclerotia often germinate by producing mycelial hyphae that can infect directly (Agrios, 2005). The disease is more severe in cool humid environments

(Agrios, 2005) and the pathogen has the ability to remain active at temperatures as low as 0 °C even if the optimum temperature for development is 18-23°C. All these survival skills of *B. cinerea* make it a serious threat for stored perishable products and a focus for significant research both pre- and postharvest (Elad et al., 2007).



**Plate 1.3:** Symptoms of grey mould infection caused by *B. cinerea* as it appears externally on eggplant, cherry tomato and pepper fruits.

## 1.4 Essential oils (EOs) as potential postharvest antimicrobial agents

### 1.4.1 What is an essential oil?

Essential oils (EOs) are volatile complex compounds derived from aromatic plant parts. Aromatic plants are generally localized in temperate to warm countries like the Mediterranean and in tropical regions (Bakkali et al., 2008). Essential oils can be present in all plant parts (flowers, buds, leaves, bracts, stems, fruits, roots) as secondary metabolites and are found in secretory structures like glandular trichomes, epidermic

cells, secretory cells and in cavities or canals (Kokkini et al., 2003; Bakkali et al., 2008). They have a protective role for plants as they are part of their defence mechanism against exogenous factors. Plants produce EOs to protect themselves against microbial infection, pests and herbivores (Tajkarimi et al., 2010).

These volatile plant extracts are oily liquids, rarely coloured and characterized by a strong odour. They are lipid soluble, soluble in organic solvents and insoluble in water (Bakkali et al., 2008). There are several methods used to extract EOs from the plants and the most commonly used is distillation (hydro-distillation or steam-distillation). Other methods for EO extraction include the use of liquid carbon dioxide, microwaves or high pressure (Burt, 2004).

EO is a mixture that constitute about 20-60 individual components at different concentrations. Commonly 2 or 3 components are in high concentration and may constitute up to 85% of the EO and the other components are present in trace amounts (Burt, 2004). EOs consist of a mixture of monoterpenes and sesquiterpene hydrocarbons and oxygenated derivatives of aldehydes, ketones, acids, alcohols and esters (Fisher and Phillips, 2008).

The total EO content, the proportion of its components and the quantitative and/or qualitative oil composition of a single species is affected by several factors. The local environment and the harvesting season appear to affect the amount and the relative proportions of the oil as the same plant produces different amount of essential oil depending on the season (seasonal variation) and climatic zone (Kokkini et al., 2003). Herbs are usually harvested when in full bloom or immediately afterwards in order to obtain good quality and quantity of EO (Burt, 2004). The oil composition may vary

among a plants' parts (different type of organ or different age) and is also dependant on the method of cultivation (Liolios et al., 2010). Furthermore, the extraction method affects the chemical profile of the extraction product in the number of molecules and in the stereochemical types of molecules extracted (Bakkali et al., 2008). In addition essential oils are volatile compounds and should be kept in airtight containers and stored in the dark in order to avoid compositional changes (Burt, 2004).

#### **1.4.2 Antimicrobial properties of plant EOs**

The role assigned to essential oils in nature suggests their antimicrobial capacity. Their medicinal properties have been recognized many years ago and they have been studied for their antibacterial, antifungal, antiviral, insecticidal, anticancer and antioxidant activity (Alexopoulos et al., 2011). It has been well established that several components of essential oils, like thymol, carvacrol, eugenol, linalool, citronellol, are biologically active with significant antimicrobial and antioxidant properties. Although these are commonly the major components of an essential oil, the minor components seem to have a critical role in an EO's biological activity (Burt, 2004). It is possible that the activity of the main components is modulated by other minor molecules by producing a synergistic effect between the components (Bakkali et al., 2008).

A main advantage of essential oils which makes them attractive to use against microorganisms is that they do not enhance antibiotic resistance as with the long-term use of synthetic antibiotics (Alexopoulos et al., 2011). In the field of food technology EOs have been tested against fungi as well as both Gram-positive and Gram-negative bacteria. Their effectiveness against microorganisms is well documented and has been reviewed extensively (Sivropoulou et al., 1996; Burt, 2004; Pramila and Dubey, 2004; Tzortzakis, 2009b; Tajkarimi et al., 2010; Solórzano-Santos and Miranda-Novales,



2012; Jayasena and Jo, 2013). It has been reported that EOs perform high antimicrobial activity *in vitro* and when applied in food a greater concentration is needed to achieve the same effect. Also, in most studies studying the action of EOs against food spoilage organisms and food borne pathogens suggest that, generally, they are slightly more active against gram-positive than gram-negative bacteria (Burt, 2004). It is noteworthy that in some cases the antifungal efficacy of the tested essential oils was similar to the levels of synthetic fungicides used in medicine and agriculture (Bishop and Reagan, 1998; Zabka et al., 2014).

#### **1.4.3 E.Os. as fresh produce preservatives**

The advantage of essential oils is their bioactivity in the vapour phase, a characteristic that makes them useful as possible fumigants for stored commodity protection (Serrano et al., 2005). Several studies reported the effectiveness of essential oil on fresh produce preservation such as sweet cherry (*Prunus avium* L.) (Serrano et al., 2005), banana (*Musa* spp.) (Anthony et al., 2003), kiwifruit (*Actinidia chinensis* L.) (Thanassouloupoulos and Yanna, 1997), grape (*Vitis vinifera* L.), pear (*Pyrus communis* L.) (Ju et al., 2000), avocado (*Persea americana* L.) (Pesis et al., 1998), strawberry (*Fragaria vesca* L.) (Tzortzakis, 2007a) and tomato (*Solanum lycopersicum* L.) fruit (Tzortzakis et al., 2011). In addition the effects of EOs on fruits quality have been studied. Some show undesirable effects on fruits quality such as residual smell or taste and phytotoxicity on EO-treated fruits (Tzortzakis, 2009b) while other reports indicated that EOs have been successful in maintaining and/or improving quality-related attributes (Wang, 2003; Tzortzakis, 2007a). Therefore EOs must be effective for control of postharvest decay and in addition should maintain or enhance fruit quality in order to be commercially applicable in postharvest technology.

### 1.5 *Origanum dictamnus* L.

*Origanum dictamnus* L. “Dittany of Crete” (Plate 1.4) is a plant species which belongs to the *Lamiaceae* (Labiatae) family. *Lamiaceae* is considered an aromatic family as it includes several medicinal herbs, some of them are native of the Cretan flora, which owe their flavours and aroma to their essential oils (Kokkini et al., 2003). *O. dictamnus* is an endemic species of the Greek island Crete and it has been used since antiquity for its medicinal properties.

Dittany was considered a “panacea” meaning medicine against all illness and among its therapeutical uses it was known for its healing properties on wounds and used for relieving pain during difficult child birth for women (Liolios et al., 2009). Dittany is still used in Crete as a traditional medicine mainly to make herbal teas, decoctions and infusions against common cold, cough and stomach discomfort, it also used as an antiseptic, digestive and diuretic (Liolios et al., 2010). The medicinal uses of the comminuted herbal substance of dittany are well documented by the final “Assessment report on *Origanum dictamnus* L., herba” for the community herbal monograph of the Committee on Herbal Medicinal Products (HMPC) of European Medicines Agency (EMA) (Chinou, 2013). Furthermore, *O. dictamnus* is characterized as a “food additive permitted for direct addition to food for human consumption” by the USA Food and Drug Administration (CFR, 2009). Among its current uses commercially dittany has been applied as a flavouring in alcoholic beverages by the ‘Martini’ company and employed in the cosmetics industry by the company under the brand name ‘Korres’.

Dittany grows wild in Crete from the sea level up to the mountains but today wild populations have decreased and rarely found in the mountains of the island. The

demand for plant material is covered by systematic cultivation in villages of Crete especially in the village of Emparos near Heraklion.



**Plate 1.4:** *O. dictamnus* L. wild plant species (Liolios et al., 2010).

*O. dictamnus* is a medicinal herb also known for its essential oil. The EO is located in the aerial parts of the plant, fresh bracts, leaves and flowers are commonly used for the extraction of EO. Leaves and other aerial parts of the plant bear a great number of glandular trichomes. Two types of glandular hairs occur in *O. dictamnus*, the capitate and the peltate hairs (Liolios et al., 2010). Even though both structures have a secretory role it seems the bulk of essential oil is produced and accumulated in the peltate hairs

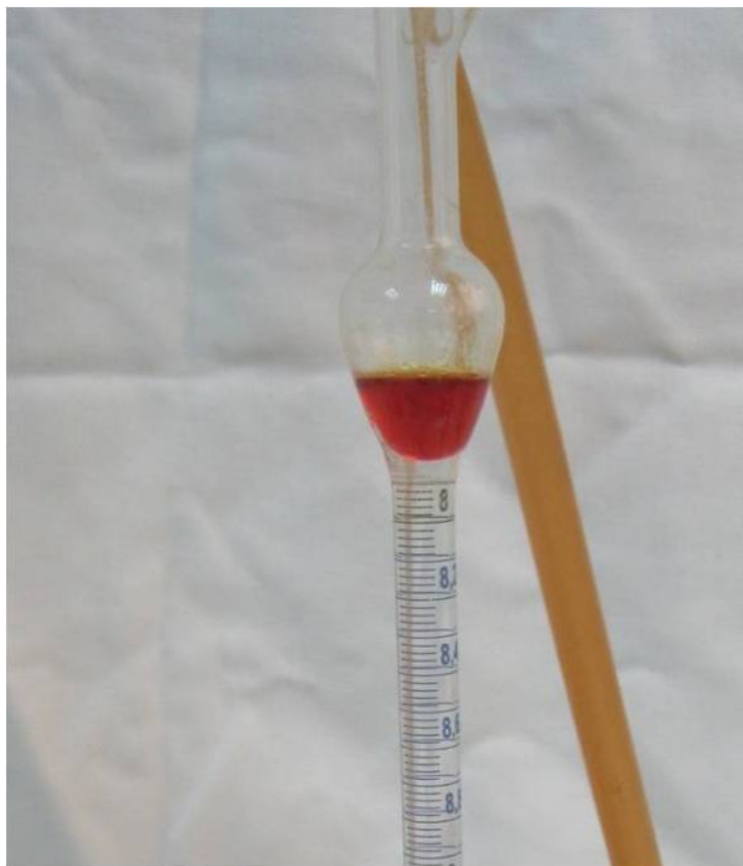
(Kokkini et al., 2003). Examining a leaf of dittany under a stereoscope one can observe these structures with EOs as indicated in “Herbs of the Labiatae” (Kokkini et al., 2003) which appear as orange dots shown in Plate 1.2. The essential oil of *O. dictamnus* is coloured orange-red (Plate 1.3). The leaves and other aerial parts of the plant are densely covered with branched non-glandular hairs which seem to have a protective role (Liolios et al., 2010).



**Plate 1.5:** Lower side of *O. dictamnus* leaf as it appears under the stereoscope.

The chemical analysis of *O. dictamnus* essential oil in the majority of data revealed carvacrol as the dominant component and  $\gamma$ -Terpinene and p-cymene in less amounts. All three are the major components of the dittany EO composition while in some cases

thymol, instead of carvacrol, was detected as the main component (Sivropoulou et al., 1996; Economakis et al., 1999; Daferera et al., 2000; Daferera et al., 2003; Liolios et al., 2010; Stavropoulou et al., 2014).



**Plate 1.6:** The essential oil of *O. dictamnus* L.

Plant extracts and/or the essential oil of dittany as well as its major components have been examined for antifungal, antibacterial, insecticidal, antioxidant and cytotoxic activity. In most studies the antimicrobial activity of *O. dictamnus* EO has been highlighted. It is suggested that the high antimicrobial activity is attributable to its rich composition in phenolic compounds like carvacrol (Liolios et al., 2010). Some research data on antimicrobial activity of dittany essential oil are outlined below:

- The essential oil of *O. dictamnus* was found to possess potent antimicrobial activities against *Erwinia carotovora* responsible for bacterial soft rot and black leg diseases of potato tubers (Vokou et al., 1993).
- Fungal development of *Penicillium digitatum* was totally inhibited at a concentration of 300 µg/mL of dittany essential oil (Daferera et al., 2000).
- A dose dependent inhibition of *Botrytis cinerea* and *Fusarium* sp. mycelial growth was caused by dittany oil while the bacterial pathogen of tomatoes, *Clavibacter michiganensis* subsp. *michiganensis*, was completely inhibited by dictamnus oils at a concentration of 100 µg/mL (Daferera et al., 2003).
- Dittany oil and pure carvacrol and thymol were found active when tested against four Gram-positive and four Gram-negative bacteria, three human pathogenic fungi as well as against the food pathogen *Listeria monocytogenes* (Liolios et al., 2009).
- *O. dictamnus* essential oil was found active against clinical strains of *Staphylococcus aureus* (Alexopoulos et al., 2011).

The desirable characteristics of *Origanum dictamnus* essential oils make it an ideal candidate for research in the area of postharvest technology, especially for control of fungal diseases.

## **1.6 Aims and objectives**

The aim of the present research project was to examine the efficacy of *Origanum dictamnus* L. essential oils (EOs) (a medicinal-aromatic plant of Crete) for the control of *B. cinerea*, a common postharvest pathogen of three economically important Cretan

vegetables, tomato, pepper and eggplant. Moreover, in order to determine the suitability of the examined EO for fresh produce preservation (maintenance and/or improvement of fruit quality), the impacts of dittany EO volatiles on quality-related attributes of these fresh produce were studied.

The experimental approach included:

- Examination and identification of the concentrations of the EO *in vitro* on artificial media, and *in situ* on the three examined vegetables for the control of *B. cinerea*.
  - Determination of effects of the volatile enrichment of *O. dictamnus* essential oil in various concentrations (0, 50, 100, 250 ppm) against *B. cinerea* development (vegetative or reproductive stage) *in vitro* and in wound-inoculated fruits.
  - Study the impacts of different times of exposure of EO volatiles on the pathogen development *in vitro* and in pepper fruits.
  - Examine the effectiveness of EO volatiles directly and via induced resistance effects on control of *B. cinerea* on these vegetables.
- Observation of the impacts of dittany EO volatiles on quality-related attributes (colour measurements, weight loss, firmness, TSS, pH, titratable acidity, respiration rates) of the examined fruits.

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## **CHAPTER 2**

### **Materials and Methods**

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## 2.1 General materials and methods

### 2.1.1 *Origanum dictamnus* essential oil

Dittany (*Origanum dictamnus* L.) fresh aerial parts were obtained from a crop in Heraklion prefecture. Plant material was collected during the early summer period (end of May) while plants were in full bloom. After harvesting, dittany plants were air-dried in a darkened well-aired, dry room, spread on work benches, in order not to affect the volatile constituents of its essential oils. Essential oil was extracted from dried material by hydro-distillation using a Clevenger apparatus for 2 hours. The essential oil was stored at 4 °C until required for experiments (Plate 2.1).



**Plate 2.1:** The Clevenger apparatus used for *O. dictamnus* essential oil extraction.

### **2.1.1.1 Chemical analysis of essential oil**

The analysis of the essential oil was performed using a Shimadzu (QP 5050A) GC, equipped with a SBP-5 capillary column (30 m, 0.25mm i.d., 0.25mm film thickness) and a quadrupole mass spectrometer as detector. The carrier gas was helium, at a rate of 0.9 mL/min. Column temperature was initially maintained for 5min at 50 °C, then gradually increased to 150 °C at a rate of 5 °C/min and kept for 10 min, and finally increased to 280 °C at 5 °C/min and held for 20 min. For GC-MS detection an electron ionization system was used with ionization energy of 70 eV. The sample was measured in a split mode procedure with a split ratio 1:42. Five  $\mu\text{L}$  of oil sample was dissolved in 1mL diethyl-ether and 1  $\mu\text{L}$  was used as an injection volume. Injector and detector (MS transfer line) temperatures were set at 230 °C and 250 °C, respectively. The scanning range was 30–700 m/z. The quantification of the components was based on the total number of fragments (total ion count) of the metabolites, as detected by the mass spectrometer. The identification of the chemical components was carried out based on the retention time of each component ( $R_t$ ) compared with those of commercially available compounds, by analysis of their mass spectra, by the use of the NIST21, NIST107, and PMWTOX2 mass spectra libraries, and by comparison with the literature data (Adams, 2007). Calculation of retention indices was performed in accordance with the work of van den Dool and Dec. Kratz (1963) in comparison to the retention times of standard hydrocarbons (C<sub>9</sub>–C<sub>25</sub>). Also, when necessary, co-injection with standard compounds was carried out.

### **2.1.2 The fungal pathogen**

*Botrytis cinerea* (BPIC 2585) was supplied by Benaki Phytopathological Institute (BPI). Isolates were aseptically sub-cultured on to triple-vented Petri dishes containing 20 mL of PDA medium. Plates were incubated in the dark at 25 °C for 1 week and cultures were stored at 4 °C until required (Tzortzakis, 2009a). All experiments (*in vitro* and *in vivo*) were prepared aseptically in a laminar flow cabinet.

### **2.1.3 Fruits**

*Solanum melongena* L. ‘Vernina’ (eggplant), *Solanum lycopersicum* L. ‘Shiren’ (cherry tomato) and *Capsicum annum* L. ‘Sammy’ (pepper) fruits were used for the *in vivo* experiments. All commodities were organic cultivated, harvested at commercial maturity and obtained from the company ‘BIANAME SA’ (Timpaki, Crete, Greece). Fruits were selected for uniformity in size, ripeness and the absence of physical defects.

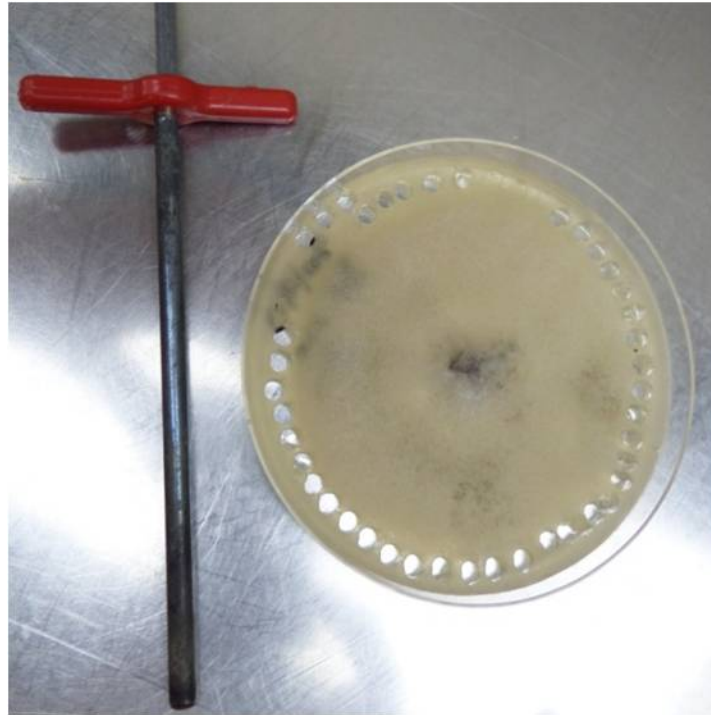
## **2.2 In vitro experimental design**

### **2.2.1 The effect of *O. dictamnus* essential oil volatile against *B. cinerea***

These experiments were designed to examine the direct impact of EO volatiles on *B. cinerea* development, at the vegetative and reproductive stage of the pathogen, and to evaluate fungicidal or fungistatic activity.

Petri dishes (90 mm diameter) containing 20 mL PDA medium were centrally inoculated with mycelial plugs (5 mm diameter), taken from the periphery of four-to-six day old fungal cultures using a sterile cork borer (Plate 2.2). Petri dishes were treated with 0, 50, 100 and 250 ppm of the volatile EO. Different times of exposure to volatiles was also examined (Tzortzakis et al., 2008) and plates were exposed to the EO for 1 day (24 h), 2 days (48 h) and continuous exposure (120 h for colony development and 14

days for conidial production, respectively). Solutions of the EO and 5% V/V Tween 20 were prepared at the desired concentrations, where controls consisted of 5% V/V Tween 20.



**Plate 2.2:** A cork borer which was used to cut mycelial plugs from the fungal culture.

The volume of 20 mL PDA was subtracted from the volume of Petri dishes (80 mL) to obtain the free volume of air inside the plates (60 mL). The free volume was used to calculate the volume of EO used to prepare solutions of the required concentrations. Sterile filter papers (3.5 \*3.5 cm) were fixed to the inside of the Petri lids and 100  $\mu$ L of the volatile solutions were pipetted onto each strip of filter paper. Inoculated plates were subsequently sealed, inverted and placed at room temperature for 2 h to allow the volatiles to vaporize inside the plates and then incubated at 12 °C ( $\pm$ 0.5) and 95% RH in climatic chambers. Nine replicates were prepared for each treatment and time of exposure (total 90 Petri dishes).

One day post-inoculation plates of each treatment exposed to the EO for 24 h were unsealed and the EO removed by replacing Petri dish lids with new ones. The Petri plates were re-sealed and stored again. This was repeated for the 48 hr treatments. Experimental design is detailed in Table 2.1.

#### **2.2.1.1 Mycelial colony development assay**

The efficacy of treatments on fungal colony development was evaluated by measuring radial growth of the colonies daily, using a digital calliper until the growth of the controls reached the edge of the Petri plates (5 days). Results are expressed as fungal colony area (in cm<sup>2</sup>) (Tzortzakis, 2009a; Tzortzakis, 2010). Petri plates were kept in storage until sporulation occurred.

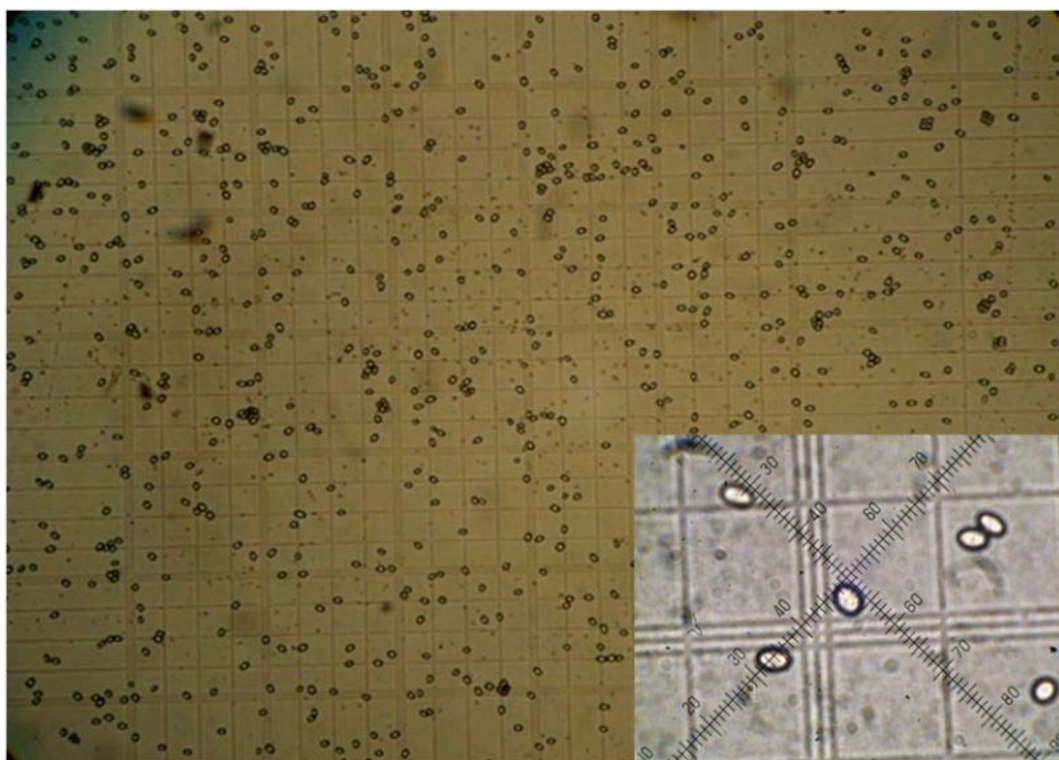
#### **2.2.1.2 Fungicidal and fungistatic evaluation**

To evaluate the fungicidal or fungistatic effects of EO volatiles, 6 days post-inoculation, at the end of the colony development assay, mycelial agar plugs were removed from the treated plates and re-inoculated on fresh PDA (3 replicates per treatment- total of 30 Petri dishes). These treatments/replicates were stored at 12 °C (±0.5) and colony development measured daily as described in Section [2.2.1.1](#).

#### **2.2.1.3 Spore production assay**

Fungal spore production was determined 14 days post-inoculation by collecting spores from treated plates (6 replicates per treatment –total of 60 Petri plates). Spores were collected by adding 5mL of 0.1% V/V Tween 80, used for better spore separation, to each plate and rubbing the surface with a sterile L-shaped spreader (3 times). The solutions were filtered using sterile gauze to remove any conidiophores or mycelium. The resulted spore suspension (approximately 15mL) from each plate was centrifuged at

room temperature at 2000 g for 5 min. The supernatant was discarded and re-centrifuged until 1mL of spore solution remained. Quantification was performed by counting spores with the use of a haemocytometer slide (Neubauer improved) (Plate 2.3) and the results expressed as number of spores/mL (Tzortzakis, 2009a).



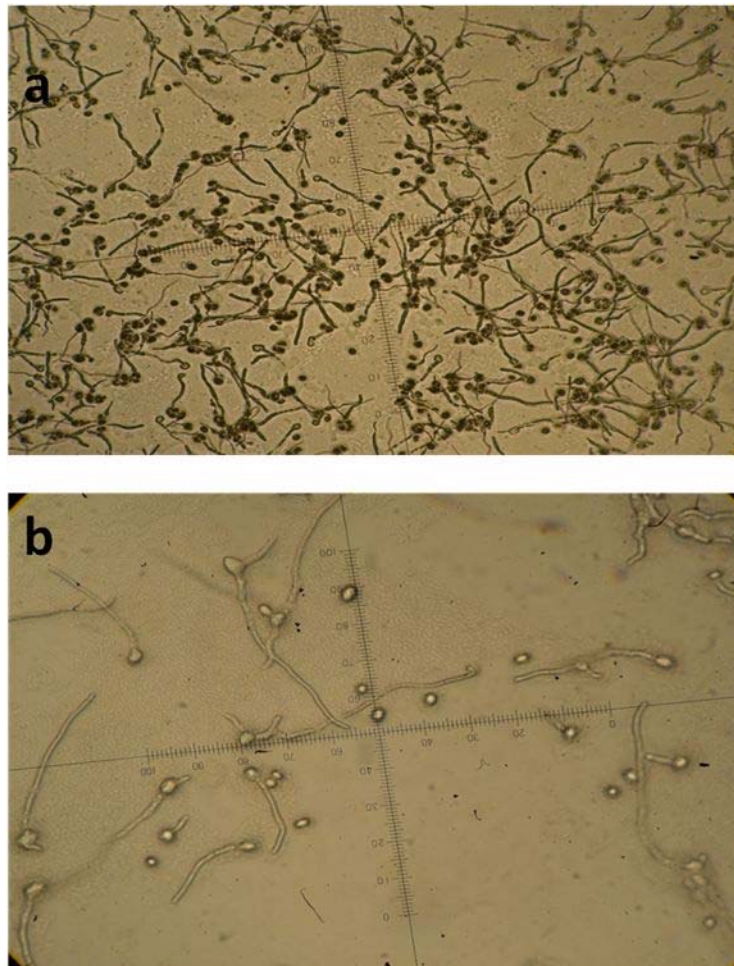
**Plate 2.3:** Conidia of *B. cinerea* as they appear under the microscope on a haemocytometer slide.

#### 2.2.1.4 Spore germination assay

In order to examine fungistatic or fungicidal effects of *O. dictamnus* EO volatiles on spore viability following treatments, 300  $\mu$ L of each spore suspension resulting from the spore production assay (Section [2.2.1.3](#)) were pipetted on to fresh thin layer PDA (5 mL). Inoculated Petri dishes (total of 60 plates) were incubated at 12 °C ( $\pm 0.5$ ) for 12-24 h until the conidia in the controls had germinated forming distinguishable germ tubes (approximately twice the size as the size of conidia). Experiments were then terminated



and germination was stopped by adding 100  $\mu$ L of Formaldehyde on filter papers fixed to the inside of the Petri dish lids. The Petri plates were sealed, inverted and left at ambient temperature overnight, then germination was evaluated (Malathrakis, 1989). Plates were placed under a microscope (Olympus CH40, Japan) and 100 spores per plate were examined randomly for the presence of germ tubes (Plate 2.4) and results were expressed in terms of the percentage of spores that had germinated (%) (Tzortzakakis, 2009a, Tzortzakakis and Economakis, 2007).



**Plate 2.4:** Germinated conidia of *B. cinerea* as they appear under the microscope a) 125x and b) 500x magnification.

**Table 2.1:** The experimental design of dittany EO volatiles against *B. cinerea* *in vitro*.

Treatment			Time of incubation at 12 °C (days)											
EO (ppm)	Rep.	exposure to EO	0	1		2		3	4	5	6		7-12	14
											rep.			
0	9		in.	c.d.		c.d.		c.d.	c.d.	c.d.	6			s.p.
											3	tr.	c.d.	
50	9	Cont	in.	c.d.		c.d.		c.d.	c.d.	c.d.	6			s.p.
											3	tr.	c.d.	
	9	48h	in.	c.d.		c.d.	c.l.	c.d.	c.d.	c.d.	6			s.p.
											3	tr.	c.d.	
	9	24h	in.	c.d.	c.l.	c.d.		c.d.	c.d.	c.d.	6			s.p.
											3	tr.	c.d.	
100	9	Cont	in.	c.d.		c.d.		c.d.	c.d.	c.d.	6			s.p.
											3	tr.	c.d.	
	9	48h	in.	c.d.		c.d.	c.l.	c.d.	c.d.	c.d.	6			s.p.
											3	tr.	c.d.	
	9	24h	in.	c.d.	c.l.	c.d.		c.d.	c.d.	c.d.	6			s.p.
											3	tr.	c.d.	
250	9	Cont.	in.	c.d.		c.d.		c.d.	c.d.	c.d.	6			s.p.
											3	tr.	c.d.	
	9	48h	in.	c.d.		c.d.	c.l.	c.d.	c.d.	c.d.	6			s.p.
											3	tr.	c.d.	
	9	24h	in.	c.d.	c.l.	c.d.		c.d.	c.d.	c.d.	6			s.p.
											3	tr.	c.d.	

Key to treatments: rep.: replicates, cont.: continuous exposure to EO, in.: inoculation, c.d.: colony diameter measurement, r.l.: replace lids to remove EO, tr.: transfer, s.p.+g.: spore production and spore germination



### **2.2.2 Pre-exposure EO of *O. dictamnus* essential oil volatiles on control of *B. cinerea* in vitro**

In this experimental setup, culture medium was pre-exposed to EO volatiles and then inoculated with the pathogen in order to examine impact of EO on fungal development (vegetative and reproductive stage). Different concentrations (0, 50, 100, 250 ppm) of EO and time of exposure (continuous and interrupted exposure) to the EO were examined.

Petri dishes containing 20 mL PDA, un-inoculated, were treated with 0, 50, 100 and 250 ppm EO as described in Section [2.2.1](#) and stored at 12 °C ( $\pm 0.5$ ) for 5 days. In order to examine effect of different times of exposure to volatiles, Petri plates of each concentration exposed to EO for 48 h were treated the 3<sup>rd</sup> day and those exposed to EO for 24 h were treated on the 4<sup>th</sup> day. There were 6 replicates for each treatment (concentration and time of exposure), total of 60 Petri plates. After 5 days of storage EO was removed by replacing Petri lids with new ones and they were then inoculated with the pathogen as described in Section [2.2.1](#) (Tzortzakis, 2007b; Tzortzakis, 2010). Inoculated Petri plates were incubated at 12 °C ( $\pm 0.5$ ) for 14 days. The experimental design is detailed in Table 2.2.

The fungal colony development was monitored daily, for 5 days, as described in Section [2.2.1.1](#) and the results expressed as fungal colony area (in cm<sup>2</sup>). The treatments and replicates were stored until sporulation occurred.

### 2.2.2.1 Spore production assay

Fungal spore production was determined 14 days post-inoculation by collecting spores from treated plates (6 replicates per treatment –total of 60 plates) as described in Section [2.2.1.3](#) and expressed as number of spores/mL.

### 2.2.2.2 Spore germination assay

Spore viability following treatments was examined as described in Section [2.2.1.4](#) (6 replicates per treatment-total of 60 samples) and results were expressed in terms of the percentage of spores that had germinated (%).

**Table 2.2:** The experimental design of effects of pre-exposed PDA medium to dittany EO volatiles against *B. cinerea* in the *in vitro*.

Treatment			Time of storage at 12 °C (days)							
EO (ppm)	Rep.	exposure to EO	0	1	2	3	4	5	6-10	19
0	9							r.l.-in.	c.d.	s.p.+g.
50	9	Cont	EO	EO	EO	EO	EO	r.l.-in.	c.d.	s.p.+g.
	9	48h				EO	EO	r.l.-in.	c.d.	s.p.+g.
	9	24h					EO	r.l.-in.	c.d.	s.p.+g.
100	9	Cont	EO	EO	EO	EO	EO	r.l.-in.	c.d.	s.p.+g.
	9	48h				EO	EO	r.l.-in.	c.d.	s.p.+g.
	9	24h					EO	r.l.-in.	c.d.	s.p.+g.
250	9	Cont.	EO	EO	EO	EO	EO	r.l.-in.	c.d.	s.p.+g.
	9	48h				EO	EO	r.l.-in.	c.d.	s.p.+g.
	9	24h					EO	r.l.-in.	c.d.	s.p.+g.

Key to treatments: rep.: replicates, cont.: continuous exposure to EO, EO: treatment with EO, in.: inoculation; r.l.: replace lids to remove EO, c.d.: colony diameter measurement, s.p.+g.: spore production and spore germination

### **2.2.3 Effect of EO volatiles on colony development of *B. cinerea* strains isolated from eggplants, cherry tomatoes and peppers grown in Crete**

In this study the efficacy of the EO against three different strains of *B. cinerea*, one from each commodity, isolated from fruits grown in Crete was examined.

They were obtained using the single spore isolation method (Choi et al., 1999). Plugs from cultures of the three strains isolated from eggplants, cherry tomatoes, peppers and the type strain (BPIC; used in all experiments) were inoculated on PDA and treated with 0, 50, 100 and 250 ppm EO as described in Section [2.2.1](#). Plates were incubated for 5 days at 12 °C, and colony development measured daily as described in Section [2.2.1.1](#) and results expressed as fungal colony area (in cm<sup>2</sup>). The objective was to compare sensitivity/tolerance of different strains of *B. cinerea* to the EO volatiles.

## **2.3 *In vivo* experimental design**

The aim of this set of experiments was to assess the potency of *O. dictamnus* EO volatile-enrichment in controlling fungal decay of fruits (eggplants, cherry tomatoes and peppers) caused by *B. cinerea* under common postharvest preservation conditions.

### **2.3.1 Effect of *O. dictamnus* essential oil volatiles on *B. cinerea* development in wound-inoculated fruits**

In order to evaluate direct impact of EO volatiles on *B. cinerea* development (vegetative and reproductive stage) the eggplants, cherry tomatoes and peppers were artificially inoculated with the pathogen and exposed to different concentrations of EO.

Fruits were surface sterilized by dipping them in 1% V/V sodium hypochlorite solution for 2 minutes followed by rinsing with sterile distilled water and left to dry in the

laminar flow cabinet. Fruits, which were equivalent in size, were randomly selected and divided into 4 batches of 12 fruits for eggplants, 24 fruits for cherry tomatoes and 6 fruits for peppers. Fruits were wounded (1 wound per fruit) using a sterile scalpel to produce uniform superficial wounds of approximately 4 mm diameter and 2 mm deep of the fruit skin (Jun et al., 2011). Eggplants were wounded in the equatorial zone, cherry tomatoes diametrically opposite the stem (calyx removed) and peppers approximately 1/3 from the apical section (where the surface is largest). Fruits were inoculated by placing a mycelia plug (3.5 mm diameter), removed from the periphery of four-to-six day old fungal culture using a sterile cork borer, into each wound (Plate 2.5). Eggplants



**Plate 2.5:** Inoculated fruits (eggplants, cherry tomatoes and peppers) placed in the containers.

were placed inside 5.4 L containers due to their large volume (4 fruits per container), while cherry tomatoes and peppers were placed into 1.6 L containers (tomatoes: 8 fruits per container, peppers: 2 fruits per container). Inoculated fruit were treated with 0, 50, 100 and 250 ppm of EO in 3 replicate containers per treatment. Solutions of EO and 5% V/V Tween 20 were prepared at the desired concentrations, where controls consisted of 5% V/V Tween 20. A graduated cylinder containing water was used to estimate fruit volumes. For each container, fruit volume (placed inside the container) was subtracted from the container volume to give the free volume of air inside it. The free volume was used to calculate the volume of EO required and appropriate solutions were prepared.

EO concentrations were applied on sterile filter papers placed in 60 mm Petri dishes without the lids, which were subsequently placed inside the containers just before the lids were closed. A filter paper moistened with water was also placed into each container to maintain high relative humidity during the storage period. Containers were placed at room temperature for 2 h to allow the volatiles to vaporize inside them and then stored at 12 °C ( $\pm 0.5$ ) in climatic chamber (Plate 2.6) for a period of 14 days (Tzortzakis et al., 2008; Tzortzakis, 2009a.). The experimental design is detailed in Table 2.3.



**Plate 2.6:** Containers stored in the climatic chamber.

**Table 2.3:** Experimental design of treatments of would inoculated fruit with *O. dictamnus* essential oil on *B. cinerea* development.

EO (ppm)	Fruit	Rep. containers	Fruits / container	Storage period at 12 ° C (days)			
				0	7	14	
0	eggplants	3	4	EO + inoculation	lesion area measurement +EO	l.a.m.	Spore production and germination
	ch.tomatoes	3	8				
	peppers	3	2				
50	eggplants	3	4			l.a.m.	
	ch.tomatoes	3	8				
	peppers	3	2				
100	eggplants	3	4			l.a.m.	
	ch.tomatoes	3	8				
	peppers	3	2				
250	eggplants	3	4			l.a.m.	
	ch.tomatoes	3	8				
	peppers	3	2				

Key to treatments: Rep.: replicates, EO: treatment with EO, l.a.m: lesion area measurement.

### 2.3.1.1 Effect of different times of exposure to EO volatiles on *B. cinerea* development in pepper fruits

In order to evaluate the impact of continuous or interrupted exposure of EO on inoculated fruits, pepper fruits were inoculated with the pathogen and treated with different concentrations of EO as described in Section [2.3.1](#) and exposed to EO for 1 day (24 h), 2 days (48 h) and continuous exposure (7 days for lesion growth and 14 days for spore production and subsequent germination) (Tzortzakakis et al., 2008). There were

2 fruits per container and 3 replicate containers per treatment (60 fruits). For the treatments of 1d exposure to EO containers were opened 24 h post-inoculation and the EO removed, containers were closed and placed back into storage. The same procedure was followed for the 2d treatments with the EO. The experimental design is detailed in Table 2.4.

**Table 2.4:** Experimental design of different time of exposure (continuous exposure, 48 and 24 hours) of EO volatiles on *B. cinerea* development in pepper fruits.

EO (ppm)	Time of exposure to EO	Rep. containers	Fruits / container	Storage period at 12 ° C (days)						
				0		1	2	7		14
0	0	3	2	EO	Inoculation			Lesion area measurement	EO	Spore production and germination
50	Cont	3	2	EO					EO	
	48h	3	2	EO			r.EO			
	24h	3	2	EO		r.EO				
100	Cont	3	2	EO					EO	
	48h	3	2	EO			r.EO			
	24h	3	2	EO		r.EO				
250	Cont	3	2	EO					EO	
	48h	3	2	EO			r.EO			
	24h	3	2	EO		r.EO				

Key to treatments: Rep.: replicates, Cont.: continuous exposure to EO, EO: treatment with EO, r.EO: remove EO.

### 2.3.1.2 Effect of EO on lesion area

Seven days post-inoculation, containers were opened and lesion area expansion on fruits was measured using a digital calliper (Plate 2.9). The shape of the lesion area on

eggplants and peppers was elliptical, probably because of the shape of fruits, therefore minor and major axes were recorded, while in cherry tomatoes lesion area was usually in circles. Data was expressed as lesion area in  $\text{cm}^2$ . Opening the containers led to changes in EO concentration inside them, therefore solutions of EO were re-applied onto new filter papers as described in Section [2.3.1](#) (only for the treatments for continuous exposure for Section [2.3.1.1](#)) and containers stored for an additional 7 days until conidia were formed. A second lesion area measurement was performed on eggplants on the 14<sup>th</sup> day, while decay on cherry tomatoes and peppers did not allow a second measurement.



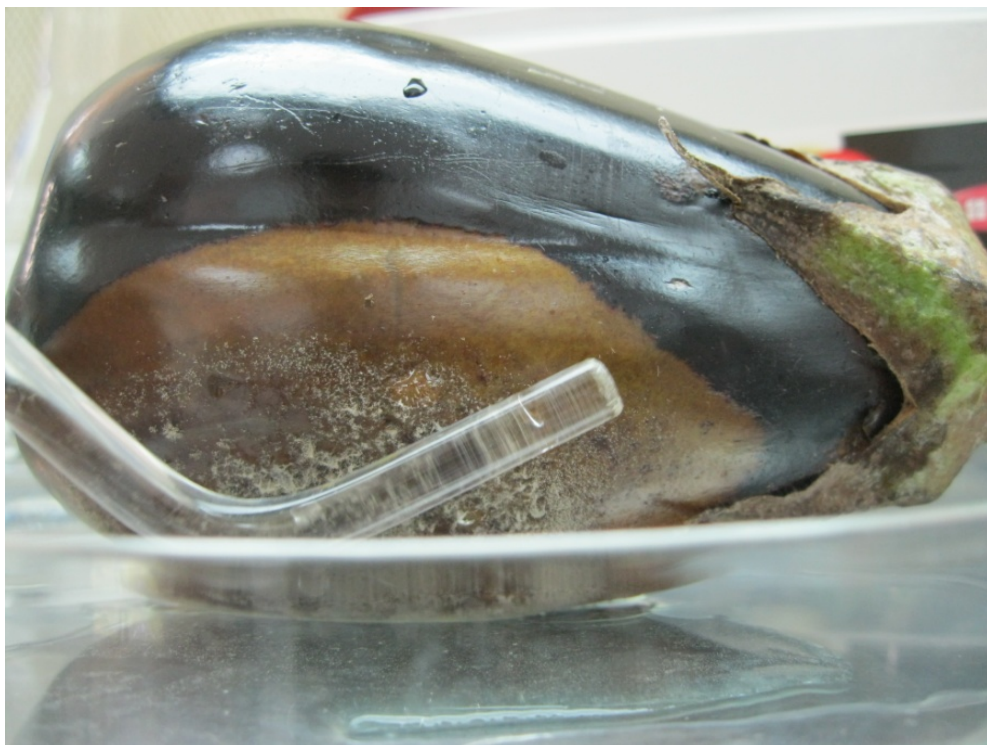
**Plate 2.7:** Measuring grey mould lesion area on inoculated pepper fruit using a digital calliper.

### 2.3.1.3 Spore production assay

Fourteen days post-inoculation, containers were brought out of refrigerated storage in order to evaluate impact of treatments on conidial production by *B. cinerea* in



inoculated fruits by quantifying spores produced per fruit. Spores were collected from inoculated fruits by placing fruits on Petri dishes (without lids, decayed surface facing the plate) and washing the decayed surface of fruits with 10 mL sterile water containing 0.1% V/V Tween 80 using a sterile L-spreader to rub the surface (3 times) (Plate 2.8). The solutions were filtered using sterile gauze to remove any conidiophores or mycelium. The resulted spore suspension (approximately 30 mL) from each fruit was centrifuged at room temperature at 2000 g for 5 min. Supernatant was discarded and re-centrifuged until 1 mL of spore solution remained. There were 6 replicate samples per treatment for peppers (total of 60 samples) and 12 replicate samples for eggplants and cherry tomatoes (48 samples per commodity). Quantification was performed using a haemocytometer slide (Neubauer improved) and results expressed as number of spores/mL.



**Plate 2.8:** Collecting conidia of *B. cinerea* from the decayed surface of eggplant fruit.

#### **2.3.1.4 Spore germination assay**

The spores obtained from the sporulation assay above were used to examine relative viability as described in Section [2.2.1.4](#) and results expressed in terms of the percentage of spores that had germinated (%).

#### **2.3.2 Induced resistance effects of *O. dictamnus* essential oil on *B. cinerea* development in wound-inoculated fruits pre-exposed to the volatile fractions**

This study was carried out to evaluate whether initial exposure to the EO, prior to infection by *B. cinerea* may result in an induced resistance response and thus facilitating control of the pathogen. This could then be compared with the results from the direct volatile exposure assays above (Tzortzakakis, 2007b).

Eggplants, cherry tomatoes and peppers were placed in containers and exposed to 0, 50, 100 and 250 ppm of EO as described in Section [2.3.1](#) and stored in climate chambers at 12 °C ( $\pm 0.5$ ) for 5 days. After this, they were opened and the EO removed (containers ventilated). The fruits were then wound inoculated as described in Section [2.3.1](#). Containers were then closed and stored again under the same conditions for 14 days. The experimental design is detailed in Table 2.5.

**Table 2.5:** Experimental design of induced resistance effects of *O. dictamnus* essential oil on *B. cinerea* development in wound-inoculated fruits pre-exposed to volatiles.

EO (ppm)	Fruit	Rep. containers	Fruits / container	Storage period at 12 ° C (days)			
				0	5	12	19
0	eggplants	3	4	EO	remove EO + inoculation	lesion area measurement	Spore production and germination
	ch.tomatoes	3	8				
	peppers	3	2				
50	eggplants	3	4				
	ch.tomatoes	3	8				
	peppers	3	2				
100	eggplants	3	4				
	ch.tomatoes	3	8				
	peppers	3	2				
250	eggplants	3	4				
	ch.tomatoes	3	8				
	peppers	3	2				

Key to treatments: Rep.: replicates, EO: treatment with EO.

### 2.3.2.1 Induced resistance effects of EO exposure on *B. cinerea* development in pre-exposed pepper fruits to volatiles

A similar procedure to that in Section 2.3.1 above was used. However, this study examined the effect of induced resistance over a continuous initial period or an interrupted exposure of EO of pepper fruits with different concentrations of EO.

The treatments were exposure for 1 day (24 h), 2 days (48 h) and continuous exposure (5 days). There were 2 fruits per container and 3 replicate containers per treatment (60

fruits) (see Section [2.3.1.1](#)). Fruits were placed into the containers and only replicates of continuous exposure treated with EO are as described in Section [2.3.1](#). All replicate containers were stored in climate chambers at 12 °C ( $\pm 0.5$ ) for 5 days. EO was added to the 48 h exposure containers on the 3<sup>rd</sup> day, while replicates of 24 h of exposure to EO treated on the 4<sup>th</sup> day of storage. After the storage period of 5 days containers were opened, EO removed (containers ventilated), and the fruits inoculated as described in Section [2.3.1](#). Containers were then closed and placed in storage for additional 14 days. The experimental design is detailed in Table 2.6.

**Table 2.6:** Experimental design of induced resistance effects of different time of EO volatiles exposure on *B. cinerea* development in pepper fruits.

EO (ppm)	Time of exposure to EO	Rep. containers	Fruits / container	Storage period at 12 °C (days)					
				0	3	4	5	12	19
0	0	3	2	EO	EO	EO	remove EO + inoculation	lesion area measurement	Spore production and germination
50	Cont	3	2	EO	EO	EO			
	48h	3	2		EO	EO			
	24h	3	2			EO			
100	Cont	3	2	EO	EO	EO			
	48h	3	2		EO	EO			
	24h	3	2			EO			
250	Cont	3	2	EO	EO	EO			
	48h	3	2		EO	EO			
	24h	3	2			EO			

Key to treatments: Rep.: replicates, Cont.: continuous exposure to EO, EO: treatment with EO.

#### **2.3.2.2 Lesion area development**

Seven days post-inoculation containers were opened and lesion area expansion on fruits recorded as described in Section [2.3.1.2](#). Containers were then stored for an additional 7 days until conidia were formed. Data are expressed as lesion area in cm<sup>2</sup>.

#### **2.3.2.3 Spore production assay**

Fourteen days post-inoculation, containers were brought out of refrigerated storage in order to evaluate impact of treatments on conidial production in inoculated fruits by quantifying spores produced per fruit as described in Section [2.3.1.3](#). Results are expressed as number of spores/mL.

#### **2.3.2.4 Spore germination assay**

Following spore production quantification, spores from the treatments were used to examine viability as described in Section [2.2.1.4](#) and results expressed in terms of the percentage of spores that had germinated (%).

### **2.4 Effect of *O. dictamnus* EO volatiles on quality - related attributes of fruits**

In order to evaluate the effect of EO volatiles on fruit quality during refrigerated storage quality parameters such as fruit weight loss and firmness, skin colour, pulp colour and calyx colour for eggplants, pulp sugar content (TSS), pH, titratable acidity and respiration rate were examined.

Healthy (non-infected) eggplants, cherry tomatoes and peppers were labelled and divided into 4 batches; the fruits were not surface sterilized. The pedicels of eggplants and peppers were cut in order to have the same length and pedicel scar and the calyces

from cherry tomatoes removed (some fruits were calyx-free after harvest) in order to have similar samples. Fruits were then weighed and their initial weight recorded, the skin colour and eggplants calyx colour evaluated (see Section [2.4.2](#) below). Fruits were placed into containers (3 eggplants / container, 8 cherry tomatoes / container, 2 peppers / container) and treated with 0, 50, 100 and 250 ppm of EO as described in Section [2.3.1](#). Three containers per treatment were placed at room temperature for 2 h to allow the volatiles to vaporize inside them and then stored at 12 °C (±0.5) in climatic chambers for a period of 7 days.

#### **2.4.1 Fruit Weight Loss**

Initial fruit weight (IW) was recorded prior to exposure to EO volatiles and after 7 days of storage. Containers were transferred to room temperature, opened, and each fruit weighed and the final weight (FW) recorded. Fruit weight loss was determined for 9 eggplants, 12 cherry tomatoes and 6 pepper fruits from each treatment and results expressed as percentage of weight loss (WL) as described below (Massolo et al., 2011):

$$WL(\%) = 100 \times \frac{IW - FW}{IW}$$

#### **2.4.2 Fruit Colour Measurements**

Fruit colour measurements were performed using a Minolta Chroma Meter CR400 (Konica Minolta, Japan) by measuring the parameters L\*, C\*, and h\* (Plate 2.9). The L\* represents lightness and ranges from 0 to 100, where 0 refers to no lightness (black) and 100 refers to maximum lightness (white). The C\* represents chroma or saturation and ranges from 0 which is completely unsaturated (neutral grey, black or white) to 100 or more which refers to very high chroma (saturation) or colour purity. The h\*

represents hue where the units are in the form of degrees° (or angles), ranging from 0° (red), 90° (yellow), 180° (green), 270° (blue) and back to 0°.

#### **2.4.2.1 Fruit Skin Colour**

In order to evaluate whether exposure to EO volatiles affects fruit skin colour during refrigerated storage the parameters  $L^*$ ,  $C^*$ , and  $h^*$  were measured for each fruit prior to EO exposure and at the end of the experiment, after 7 days of exposure to EO. Fruits of equal colour values before treatments were selected. Skin colour was determined for 9 eggplants, 12 cherry tomatoes and 6 pepper fruits for each treatment and three readings were made per fruit at the equatorial zone.

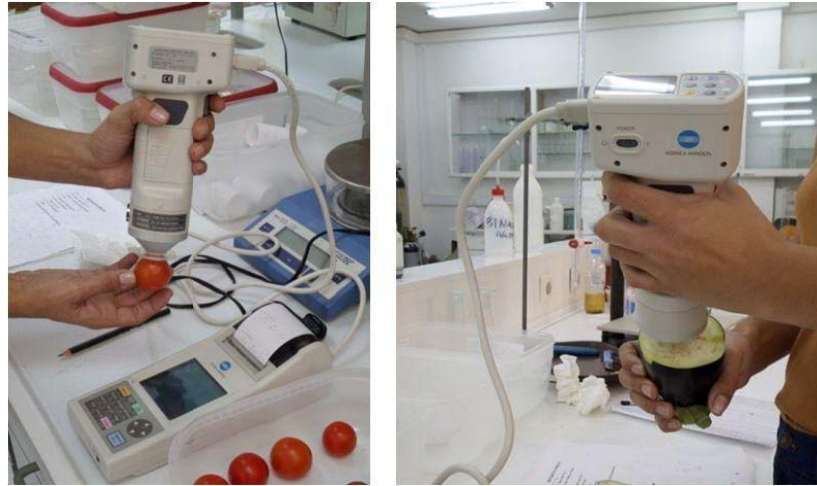
#### **2.4.2.2 Eggplant Calyx Colour**

Fresh green calyx is considered to be an important quality index for eggplants (Massolo et al., 2011). Therefore the severity of calyx damage after 7 days exposure to EO during refrigerated storage was evaluated by measuring the parameters  $L^*$ ,  $C^*$ , and  $h^*$  using a Minolta Chroma Meter CR400 as described above. Nine fruits were evaluated for each treatment and two readings were made on each fruit on opposite sides of the calyx (n=36).

#### **2.4.2.3 Eggplant Pulp Colour**

Quality attributes for eggplant fruits also include absence of seed or pulp browning (Concellón et al., 2007; Massolo et al., 2011). In order to evaluate pulp lightness of fruit tissue after 7 days of exposure to EO, each eggplant was cut horizontal in the equatorial zone in two pieces and pulp colour measured immediately using a Minolta Chroma Meter CR400, as described above, by measuring the parameter  $L^*$  (0 = black and 100 =

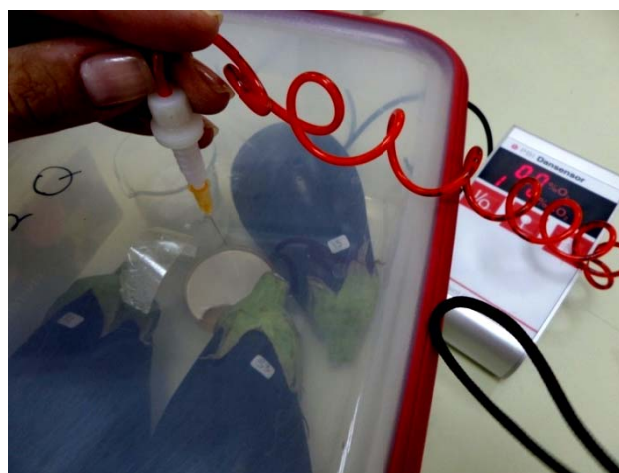
white) (Plate 2.9). Nine fruits were evaluated for each treatment and three readings taken for each fruit.



**Plate 2.9:** Measurements of the skin colour of cherry tomato and the pulp colour of eggplant fruit using a Minolta Chroma Meter.

#### 2.4.3 Respiration Rate

In order to evaluate whether the respiration of fruits was affected by the presence of EOs during refrigerated storage (7 days), CO<sub>2</sub> production was evaluated using a Handheld Gas Analyser, Check Point O<sub>2</sub>/CO<sub>2</sub>, PBI (Dansensor, Denmark) (Plate 2.10).



**Plate 2.10:** Measuring CO<sub>2</sub> production inside a shield container with fruits using a Handheld Gas Analyser.



Three containers were evaluated per treatment and 3 readings taken per container every 24 h during the storage period. Results were expressed as  $\text{cm}^3\text{CO}_2 \text{ Kg}^{-1}\text{h}^{-1}$  (Fonseca et al., 2002) as described below:

$$R_{\text{CO}_2} = \frac{(y_{\text{CO}_2}^{tf} - y_{\text{CO}_2}^{ti}) \times V}{100 \times M \times (tf - ti)}$$

where:

R=respiration rate,  $\text{cm}^3\text{CO}_2\text{Kg}^{-1}\text{h}^{-1}$

y=volumetric concentration, % V/V

V=free volume,  $\text{cm}^3$

M=mass, Kg

t=time, h

f=final

i=initial

#### 2.4.4 Fruit Firmness

Fruit firmness was evaluated for each commodity 7 days post-storage using a penetrometer Chatillon DPP Dial Push-Pull Gauze (Chatillon, France). Firmness was measured on 9 eggplants per treatment at 2 opposite points (skin removed) on the equatorial zone of each fruit by applying a flat plunger of 8 mm in diameter (Plate 2.11). Fruit firmness was measured at 1 point (skin removed) on 12 cherry tomatoes per treatment by applying a flat plunger of 5 mm in diameter. While, firmness measurements on 6 peppers per treatment were carried out on rectangular fruit pieces (approximately 20 \* 40 mm) taken from the two opposite equatorial sides of the same fruit by applying a flat plunger of 8mm in diameter (Manolopoulou et al., 2010). The

force required to compress the pericarp per fruit was recorded and expressed as  $\text{Kg cm}^{-2}$  (Tzortzakis, 2007a).



**Plate 2.11:** The penetrometer Chatillon DPP Dial Push-Pull Gauge used for fruit firmness measurements.

#### **2.4.5 Total Soluble Solids (TSS), pH, Titratable Acidity (TA)**

Fruit tissue from each commodity grinded in a mill in order to homogenize and then hand compressed using a cheese cloth filter to obtain fruit juice. The resulting fruit juice was used to determine total soluble solids (TSS) concentration of treated fruits using a digital refractometer PR-1 (ATAGO, Japan) at 20 °C and results were expressed as the mean (%) of °Brix. The pH of fruit juice was determined using a standard pH-meter (InoLAB, pH level 2, WTW, Germany). Titratable acidity (TA) was measured titrimetrically using fruit juice samples diluted in distilled water and titrated with 0.1 N NaOH up to 8.2 end point with a pH-meter. The reported values were expressed in

terms of citric acid percentage for the three commodities (Sadler and Murphy, 2010) as described below:

$$\% \text{acid(wt/vol)} = \frac{N \times V_1 \times Eq\ wt}{V_2 \times 1000} \times 100$$

where :

N=normality of titrant

$V_1$  = volume of titrant (mL)

Eq. wt. = equivalent weight of predominant acid (mg/mEq)

$V_2$  = volume of sample (mL)

1000 = factor relating mg to grams (mg/g)

## 2.5 Statistical Analysis

Data were first tested for normality, and then subjected to analysis of variance (ANOVA). Sources of variation were time of storage and treatments. Significant differences between mean values were determined using Duncan's Multiple Range test ( $P= 0.05$ ) following one-way ANOVA. Significant differences on percentage values (spore germination) were logarithmic transformed prior using ANOVA. Statistical analyses were performed using SPSS (SPSS Inc., Chicago, USA) and graphs were produced using Graph Pad Prism 5.01 (Graph Pad Inc., San Diego, USA).

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## **CHAPTER 3**

### **Results and Discussion**

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### 3.1 Chemical analysis of the essential oil

Dittany EO was extracted from plant material by hydro-distillation and the chemical analysis performed by the GC-MS method. The analysis of the essential oil of dittany allowed the identification of 24 components. Oxygenated monoterpenes (72.86 %) were the predominant fraction of the oil in which carvacrol (70.01 %) was the dominant component and p-cymene (12.65 %) and  $\gamma$ -Terpinene (7.11 %) followed in amounts as major components of the EO. The chemical and percentage composition of the major components from the essential oil of dittany is shown in Table 3.1.

**Table 3.1:** The chemical and percentage composition of the major components from the essential oil of *Origanum dictamnus* L.

No	Compound	R.I. <sup>b</sup>	Retention time	Percentage (%) Composition
1	$\alpha$ -Thujene	930	9.507	0.68
2	$\alpha$ -Pinene <sup>a</sup>	939	9.746	0.56
3	Sabinene	975	11.253	0.06
4	$\beta$ -Pinene <sup>a</sup>	979	11.353	0.10
5	1-Octen-3-ol	979	11.530	0.10
6	$\beta$ -Myrcene	990	11.933	1.12
7	$\alpha$ -Phellandrene	1002	12.402	0.10
8	$\alpha$ -Terpinene	1017	12.855	1.48
9	p-cymene <sup>a</sup>	1024	13.163	12.65
10	Limonene <sup>a</sup>	1029	13.302	0.37
11	$\gamma$ -Terpinene <sup>a</sup>	1059	14.400	7.11
12	cis-Sabinene hydrate	1070	14.701	0.72
13	Linalool <sup>a</sup>	1096	15.841	0.54
14	Terpinen-4-ol	1177	18.479	0.29
15	Thymoquinone	1252	20.816	0.19
16	Thymol <sup>a</sup>	1290	22.007	0.11
17	Carvacrol <sup>a</sup>	1299	22.382	70.01
18	$\alpha$ -Cubenene	1348	23.768	0.08
19	$\alpha$ -Copaene	1376	24.565	0.58
20	$\beta$ -Caryophyllene	1419	25.883	1.39
21	$\beta$ -Bisabolene	1505	27.302	0.14
22	$\delta$ -Cadinene	1523	29.561	0.29
23	Thymohydro quinone	1555	30.761	1.00
24	Caryophyllene oxide	1583	32.548	0.20
<b>Total (%)</b>				<b>99.87</b>
<i>Monoterpene hydrocarbons</i>				24.33
<i>Oxygenated monoterpenes</i>				72.86
<i>Sesquiterpene hydrocarbons</i>				2.68

a) Identification by comparison of retention times and co-injection with authentic compound.

b) R.I. (Retention Indices) from experimental using a SBP-5 column using a homologous series of n-alkanes (C9-C25).

## **3.2 Impacts of *O. dictamnus* EO volatiles on *B. cinerea* development *in vitro***

PDA plates were inoculated with the pathogen or pre-exposed to volatiles and then inoculated with the pathogen and then treated with dittany EO. The impacts of concentration (0, 50, 100 or 250 ppm) and duration of exposure to volatiles on pathogen development (vegetative or reproduction phase) were evaluated. Furthermore, fungicidal or fungistatic effects of dittany volatiles were examined by re-inoculating EO-treated mycelial agar plugs onto fresh PDA.

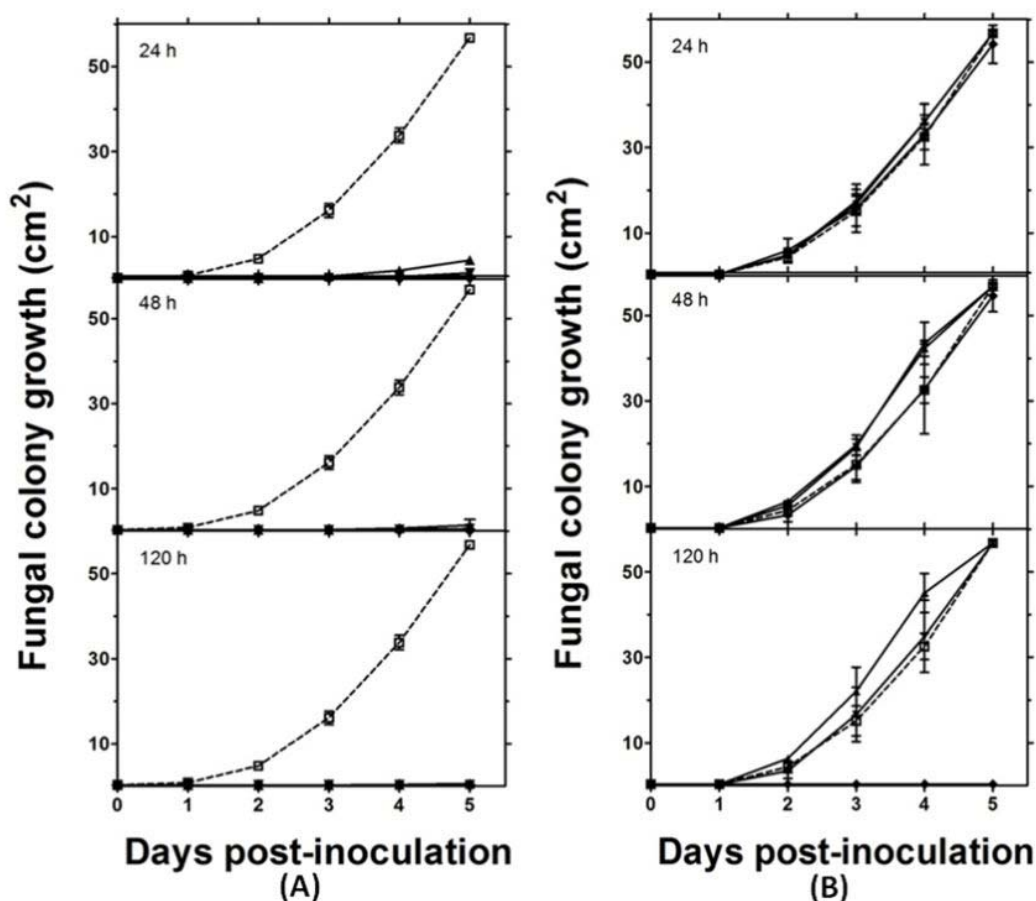
### **3.2.1 Effects of dittany EO volatiles on the colony development of the pathogen**

The EO volatiles effect on fungal colony development, on inoculated PDA plates treated with the EO at different concentrations for 24 or 48 hours (interrupted exposure) and 120 hours (continuous exposure) were evaluated. Treatment of *B. cinerea* with dittany oil enrichment resulted in a significant ( $P < 0.05$ ) reduction on subsequent mycelial colony (vegetative phase) development (between 92 and 99%). This occurred even after a short (24 h) exposure with the EO. Fungal colony growth was inhibited with the application of 50 ppm EO (continuous or interrupted exposure) with a higher level of inhibition as treatment concentration as increased (Figure 3.1).

The inhibitory effects were maintained for up to 14 days post-inoculation especially with the continuous application of 100 and 250 ppm of EO. Interrupted exposure (24 or 48h) of EO led to a temporary inhibition of fungal colony growth, dependent on the time and dose of the application (Figure 3.2).

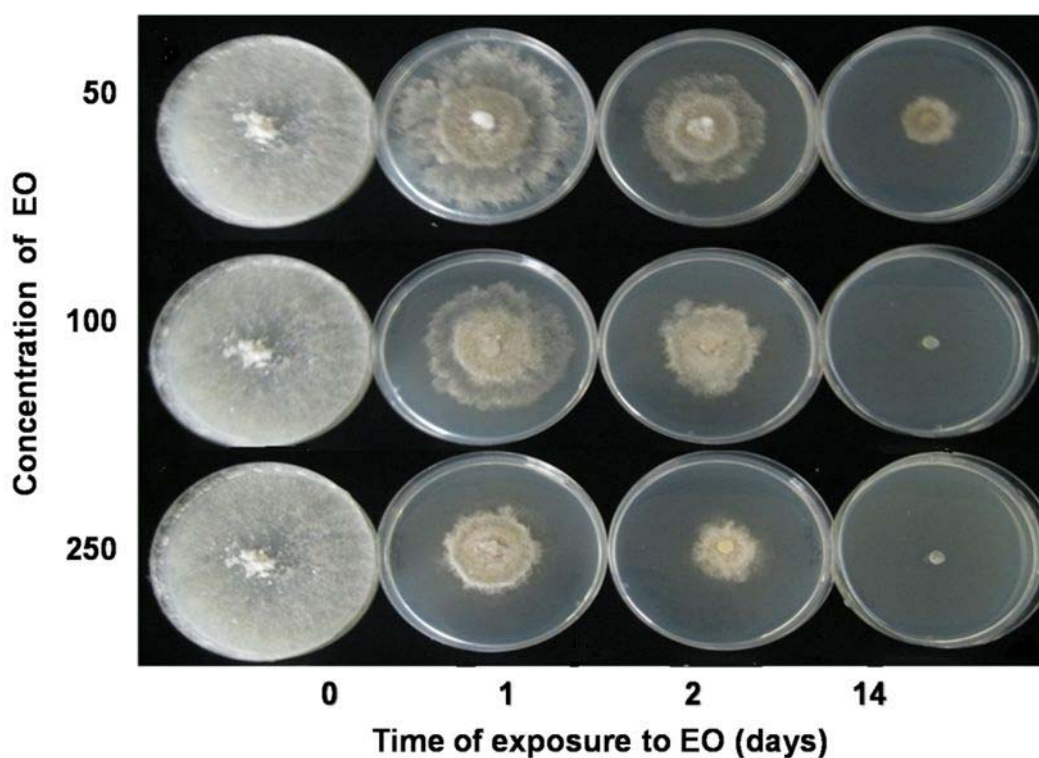
Treated inoculums, taken from the above plates, that transferred to fresh PDA the 6<sup>th</sup> day of application developed equally well in EO-treated and control-treated media implicating the fungistatic effect of dittany EO. Fungicidal effects observed with the 250 ppm application of dittany volatiles (Figure 3.1b).

Similarly, in pre-exposed PDA to different EO concentrations for 24, 48 or 120 h to dittany volatiles, vapour enrichment suppressed fungal colony growth after vapour exposure, independently of concentration and duration of EO exposure (Figure 3.3).



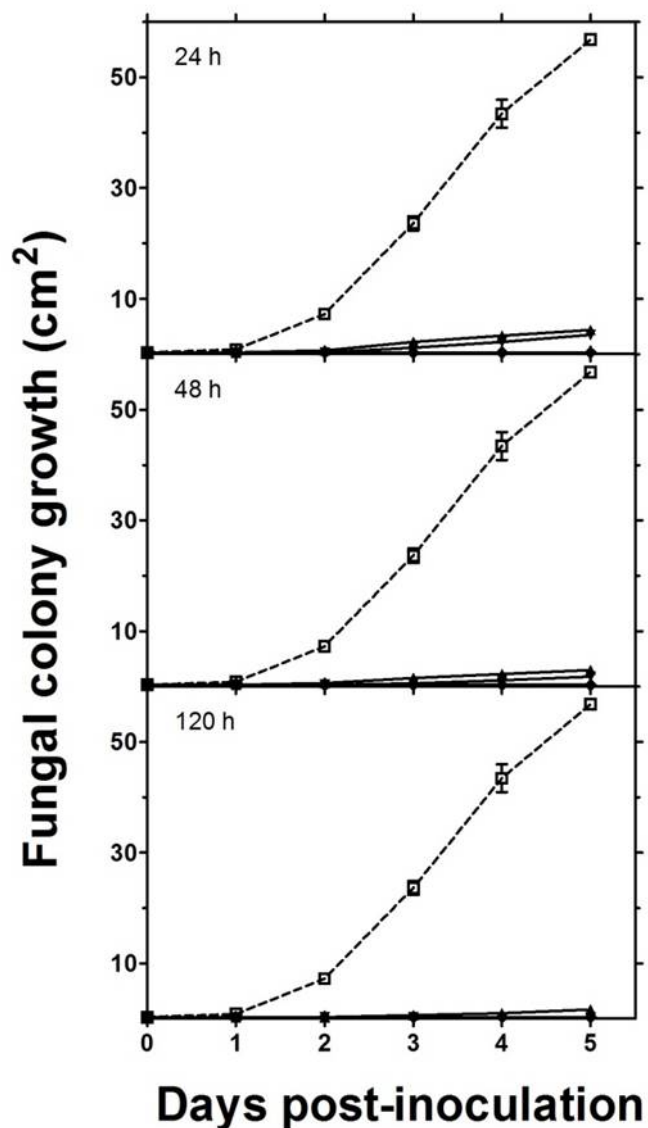
**Figure 3.1:** Impacts of dittany essential oil-enrichment (Control: --□--) or volatiles on colony development (cm<sup>2</sup>) of grey mould (*B. cinerea*) raised and exposed to dittany vapours on PDA (A) during volatiles exposure or (B) following transfer to fresh PDA medium; Key to treatments: 50 ppm (—▲—), 100 ppm (—▼—), 250 ppm (—◆—). Plates were maintained at 12°C and 95% RH. Values represent mean (± SE) of measurements made on six (A) and three (B) independent plates per treatment.

*O. dictamnus* vapour oil exhibited inhibitory effects on grey mould colony development in both types of treatment (interrupted or continuous) with a higher inhibitory effect in the continuous exposure treatment (120 h). Furthermore, fungal colony development (vegetative and reproductive phase) was suppressed completely with the continuous application of 100 and 250 ppm EO (Figure 3.4). *In vitro* studies of oregano (*Origanum vulgare* L.), thyme (*Thymus capitatus* L.), lemongrass (*Cymbopogon citratus* L.), and cilantro (*Coriandrum sativum* L.) with vapours (500–1000  $\mu\text{L/L}$ ) previously showed complete growth inhibition of *B. cinerea* and *Alternaria arborescens* (Plotto et al., 2003).

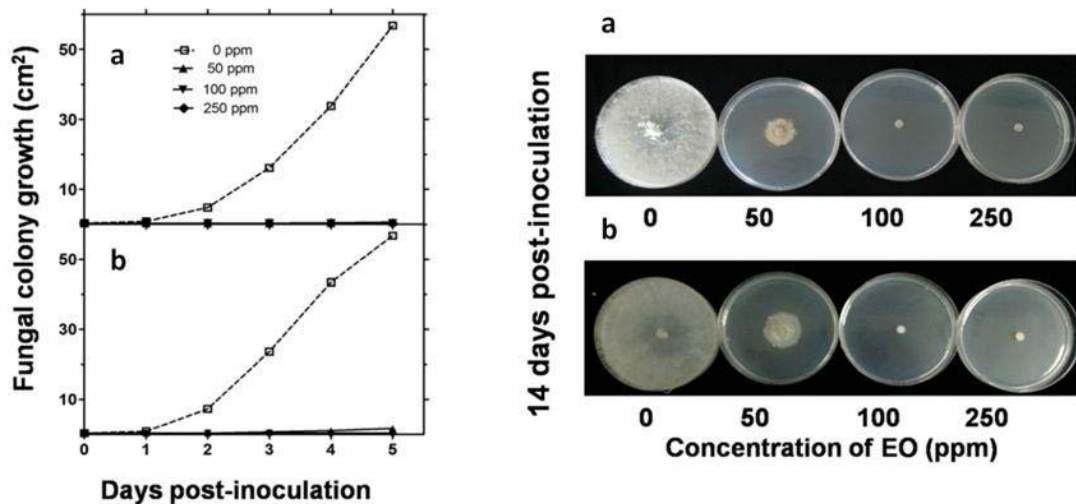


**Figure 3.2:** Impacts of concentration (0, 50, 100, 250 ppm) and exposure time (24, 48, 120 h) of dittany EO volatiles on *B. cinerea* colony development *in vitro* 14 days post-inoculation.





**Figure 3.3:** Impacts of dittany essential oil-enrichment (Control: --□--) or volatiles on colony development (cm<sup>2</sup>) of grey mould (*B. cinerea*) on pre-exposed PDA to dittany vapours and then inoculated with fungus. Key to treatments: 50 ppm (—▲—), 100 ppm (—▼—), 250 ppm (—□—). Plates were maintained throughout at 12 °C and 95 % RH. Values represent mean (± SE) of measurements made on six independent plates per treatment.



**Figure 3.4:** Impacts of dittany EO volatiles on *B. cinerea* colony development. a) Direct continuous exposure effects of EO and b) effects of continuous pre-exposure PDA to EO on fungal colony development 5 and 14 days post-inoculation.

### 3.2.2 Effects of dittany EO volatiles on pathogen's sporulation and spore viability

The effect of volatiles on *B. cinerea* sporulation and spore viability following treatments detailed above were examined for up to 14 days post-exposure.

The continuous direct impact of the EO on sporulation and spore germination (reproductive phase) *in vitro* revealed that both phases were significantly ( $P < 0.05$ ) inhibited when compared with equivalent control plates (Table 3.2). Spore production by *B. cinerea* was completely inhibited in both pre-exposed or continuously exposed treatments with 100 or 250 ppm of EO. A significant inhibition was also observed in the lowest (50 ppm) EO concentration in the pre-exposed PDA treatments. Spore germination could not be measured in the 100 and 250 ppm EO treatments because no spores were produced. Previous studies have reported that the sporulation of *Colletotrichum coccodes*, *B. cinerea*, *Cladosporium*

*herbarum*, *Rhizopus stolonifer*, and *Aspergillus niger* was inhibited by other EOs of lemongrass or cinnamon oil (Tzortzakis and Economakis, 2007; Tzortzakis, 2009a).

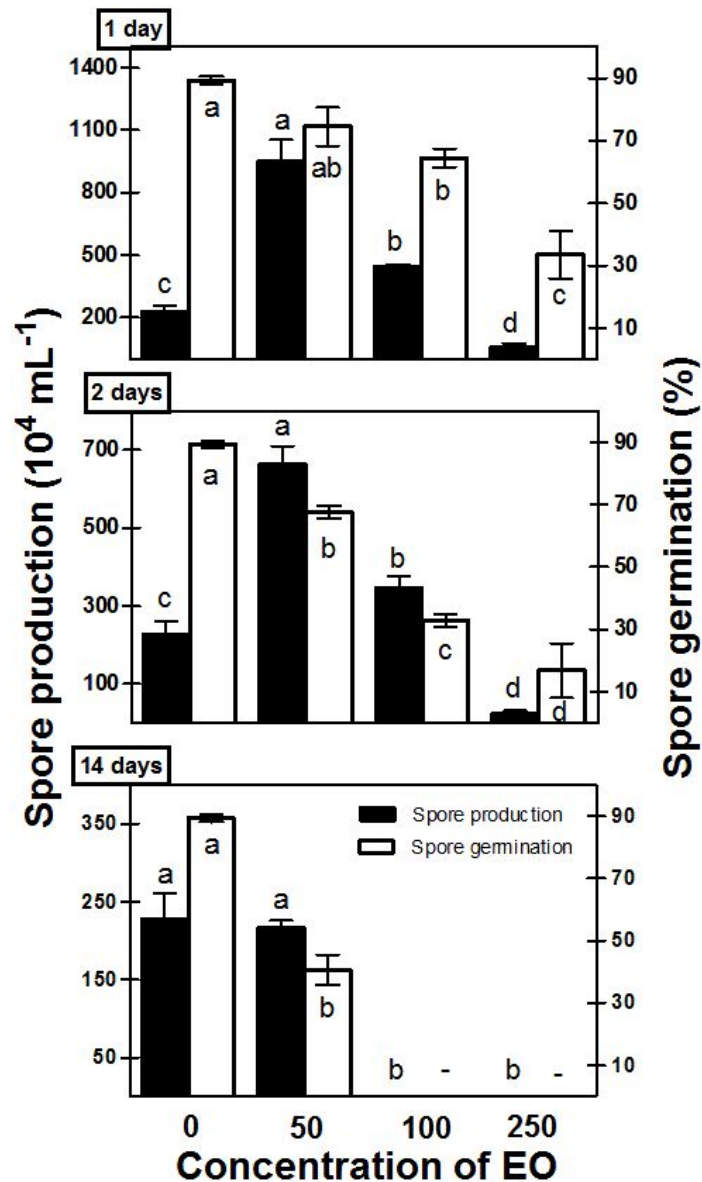
**Table 3.2:** Effects of dittany EO volatiles (A) sustained direct impact and (B) pre- exposed PDA application on *B. cinerea* sporulation and spore germination *in vitro*. In each column mean values (n=6) of plates for the individual vapour enrichment followed by the same letter do not differ significantly at  $P = 0.05$  according to Duncan's range test.

EO (ppm)	(A)		(B)	
	Spore production (10 <sup>5</sup> /mL)	Spore germination (%)	Spore production (10 <sup>5</sup> /mL)	Spore germination (%)
0	22.70 a	89.33 a	33.25 a	98.66 a
50	21.57 a	40.66 b	16.31 b	97.83 a
100	0.00 b	-	0.00 c	-
250	0.00 b	-	0.00 c	-

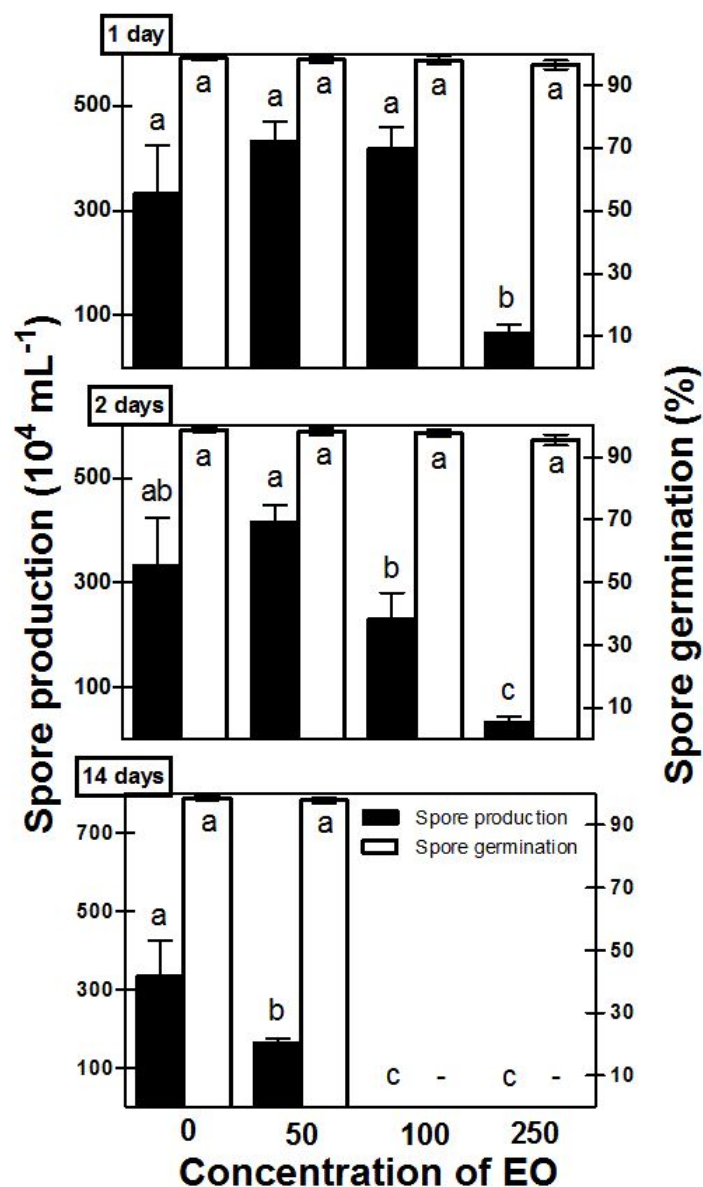
- : implicated that spore germination could not been measured as spores have been not produced

Significant reduction in fungal sporulation and spore germination was found in the 250 ppm dittany EO volatile treatment, even when the pathogen was exposed for 24 or 48 hrs only. In contrast, an interrupted application of doses < 250 ppm had very little effect. Indeed, at 50 and 100 ppm EO volatile treatment short term exposure resulted in an increase in sporulation when compared to the controls (Figure 3.5).

In pre-exposed PDA the interrupted (1 or 2 days) application of 250 ppm of EO volatiles reduced *B. cinerea* sporulation, although no effects on spore viability were observed. Lower EO concentrations had limited effects on control of *B. cinerea* in the short term exposure treatments (Figure 3.6).



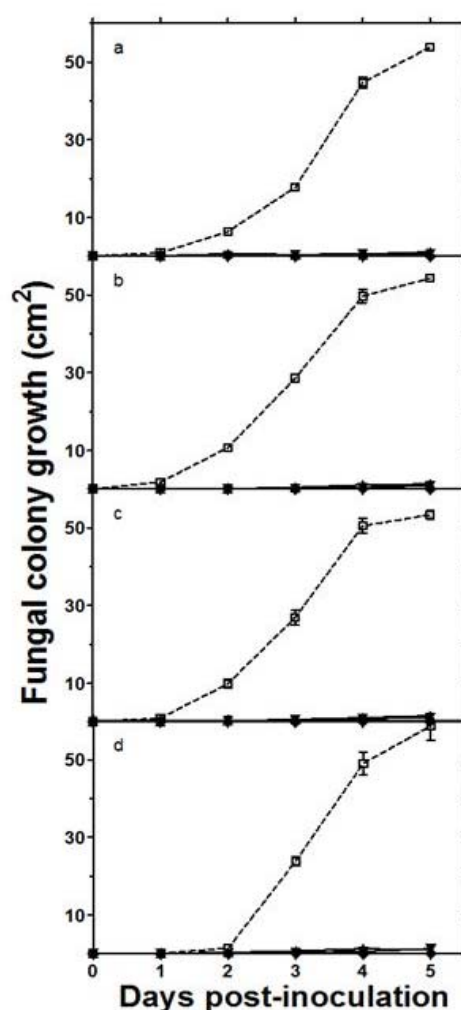
**Figure 3.5:** Impacts of concentration and time of exposure (1,2 or 14 days) of dittany EO volatiles on *B. cinerea* sporulation and spore germination *in vitro*. Values represent mean ( $\pm$  SE) of measurements made on six independent plates per treatment. Treatments followed by the same letter do not differ significantly at  $P=0.05$  according to Duncan's Range Test (- : implicated that spore germination could not be measured as spores have been not produced).



**Figure 3.6:** Impacts of concentration and time of exposure of dittany EO volatiles, pre-exposed PDA application, on *B. cinerea* sporulation and spore germination *in vitro*. Values represent mean ( $\pm$  SE) of measurements made on six independent plates per treatment. Treatments followed by the same letter do not differ significantly at  $P=0.05$  according to Duncan's Range Test (- : implicated that spore germination could not been measured as spores have been not produced).

### 3.2.3 Impacts of dittany EO on colony development of *B. cinerea* strains isolated from eggplants, cherry tomatoes and peppers grown in Crete

The three strains of *B. cinerea* isolated from peppers, cherry tomatoes and eggplants grown in Crete behaved similarly to the type strain (BPIC; used in all experiments) when treated with 0, 50, 100 or 250 ppm of the EO volatiles (Figure 3.7). This suggests similar efficacy of the EO against a range of strains of the same species.



**Figure 3.7:** Impacts of dittany essential oil [(Control: --□--), 50 ppm (—▲—), 100 ppm (—▼—), 250 ppm (—◆—)] on colony development (cm<sup>2</sup>) of 3 strains of *B. cinerea* isolated from a) pepper fruits, b) cherry tomato fruits, c) eggplant fruits and d) BPIC 2585 supplied by BPI. Plates were maintained throughout at 12 °C and 95 % RH. Values represent mean (± SE) of measurements made on three independent plates per treatment.

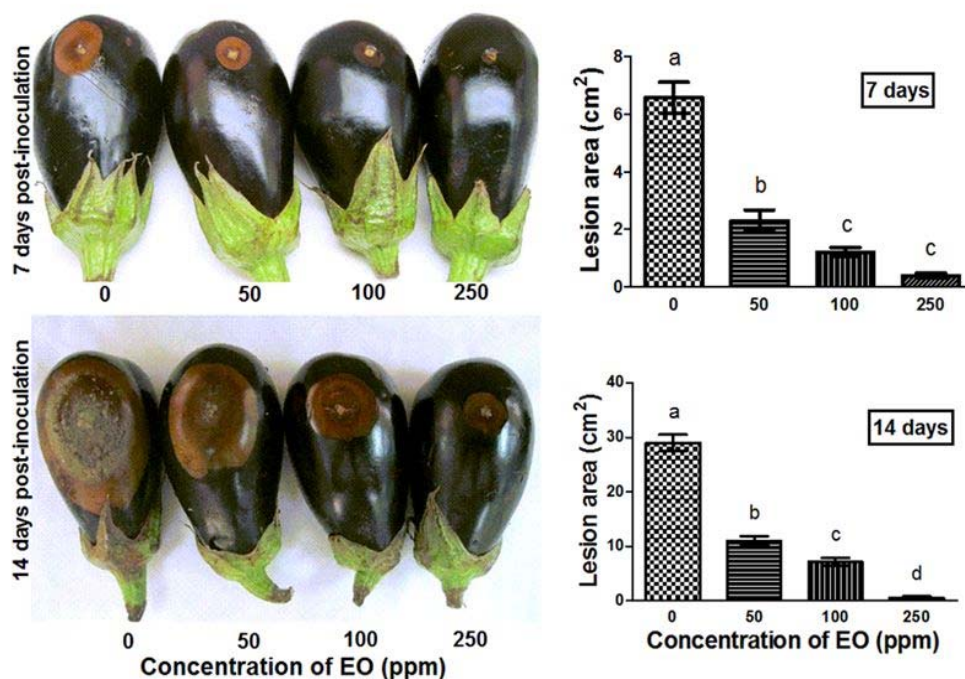
### **3.3 Impacts of *O. dictamnus* EO volatiles on *B. cinerea* development in wound-inoculated fruits**

In this experiment, eggplant, cherry tomatoes and pepper fruits inoculated with *B. cinerea* and exposed to different EO concentrations (0, 50, 100, 250 ppm) or pre-exposed to volatiles for 5 days before fungal inoculation were evaluated. In order to examine effects of EO on grey mould disease development, fruit lesion expansion was evaluated 7 or 14 days post-inoculation. Sporulation and spore viability of developing lesions were evaluated 14 days post-inoculation.

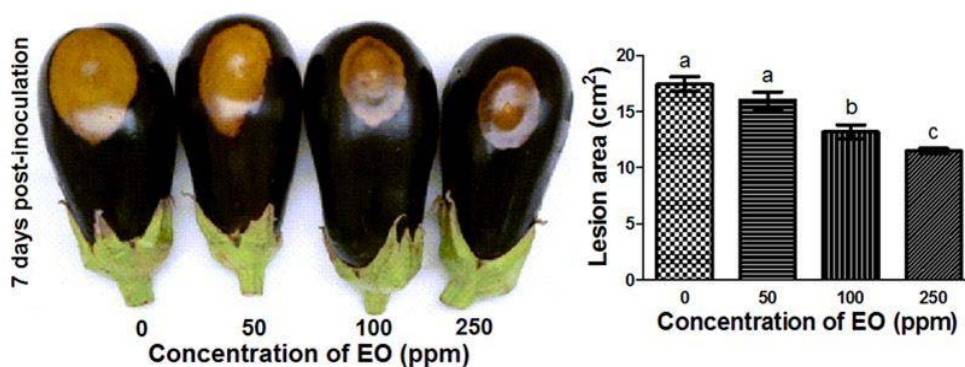
#### **3.3.1 Effects of dittany volatiles on pathogen's development in wound-inoculated eggplants**

In inoculated eggplant fruits exposure to different concentrations of the EOs resulted in a suppression of fungal lesion expansion on the fruits. The pathogen development was significantly reduced in the 50 ppm treatment 7 days post-inoculation. Increasing EO concentration resulted in better fungal inhibition. After 14 days, the statistical analyses by ANOVA revealed that the inhibitory effects was maintained 14 days post-inoculation with a significant reduction in lesion size compared to the controls (Figure 3.8).

Interestingly, pre-exposure of fruits to volatiles before pathogen inoculation, showed a better inhibition of grey mould lesions with a significant reduction in the 100 and 250 ppm treatments 7 days post-inoculation (Figure 3.9). These findings implicate possible induced resistance of fruits against the pathogen initiated by the treatment with the EO volatiles. Unfortunately, significant decay 14 days post-inoculation, especially in the control treatments, meant that a second measurement of the pre-treated fruits could not be made.



**Figure 3.8:** Impacts of dittany EO volatiles on eggplant lesion area (cm<sup>2</sup>) of grey mould (*B. cinerea*) 7 and 14 days post-inoculation. Values represent mean ( $\pm$ SE) of measurements made on twelve independent fruits per treatment. Treatments followed by the same letter do not differ significantly at  $P=0.05$  according to Duncan's Range Test.



**Figure 3.9:** Impacts of dittany EO volatiles on lesion area (cm<sup>2</sup>) of grey mould (*B. cinerea*), in pre-exposed fruits to volatiles (induced resistance effects), 7 days post-inoculation. Values represent mean ( $\pm$ SE) of measurements made on twelve independent fruits per treatment. Treatments followed by the same letter do not differ significantly at  $P=0.05$  according to Duncan's Range Test.



Fourteen days post-inoculation fungal sporulation was evaluated in treated eggplants. *B. cinerea* sporulation was reduced during exposure of fruits to different concentrations of EO volatiles. The 50 ppm treatment significantly reduced fungal spore production while no spores were produced in the 250 ppm concentration. The viability of spores from the 100 ppm treatment resulted in decreased spore viability suggesting that the spores produced on the fruit surface were malformed, indicating physiological effects on the sporulation phase. In the eggplants pre-exposed to the EOs before fungal inoculation, ANOVA revealed significant suppressed of spore production in all the treatments with greatest reduction in the maximum exposure concentration of 250 ppm (Table 3.3). However, the viability of spores which were produced were unaffected by the treatments.

**Table 3.3:** Impacts of dittany EO volatiles (A) sustained-direct effect and (B) induced-resistance effect application on *B. cinerea* sporulation and spore germination on eggplant fruits 14 days post-inoculation. In each column mean values (n=12) followed by the same letter do not differ significantly at P=0.05 according to Duncan's Range Test.

EO (ppm)	(A)		(B)	
	Spore production (10 <sup>5</sup> /mL)	Spore germination (%)	Spore production (10 <sup>5</sup> /mL)	Spore germination (%)
0	69.94 a	99.00 a	51.67 a	99.08 a
50	1.46 b	98.16 a	16.67 b	97.41 a
100	0.06 b	96.58 b	10.77 c	97.00 a
250	0.00 b	-	5.49 d	97.41 a

- : implicated that spore germination could not been measured as spores have been not produced

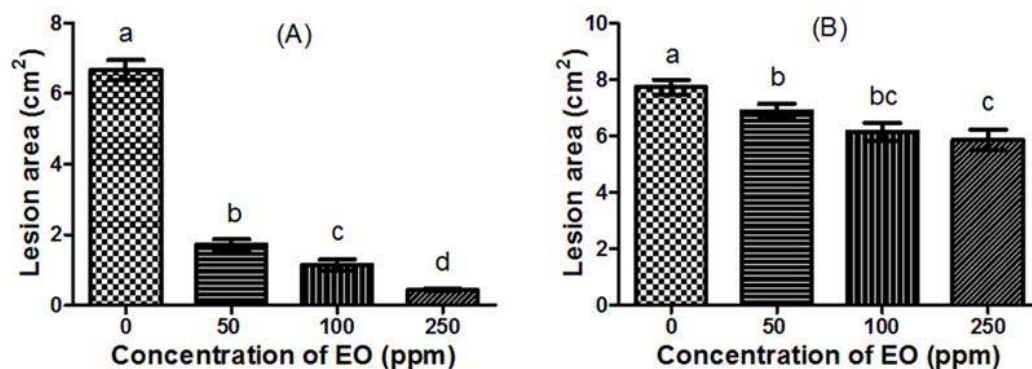
### **3.3.2 Effects of dittany volatiles on *B. cinerea* development in wound-inoculated cherry tomato fruits**

Cherry tomatoes were inoculated with *B. cinerea* and treated with 0, 50, 100 and 250 ppm of dittany EO volatiles. Following 7 days of exposure to the EOs, fungal lesion measurements on fruits revealed that pathogen development was reduced in EO-treated fruits compared to the controls. The application of 50 ppm of EO led to significant reduction while greater effects occurred at the highest concentration (250 ppm) (see Figures 3.10, 3.11).

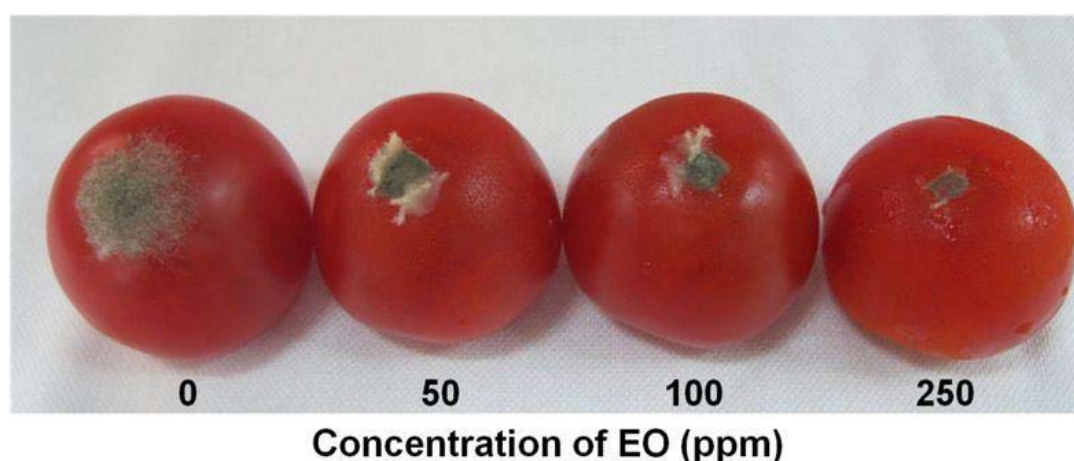
Additionally, there appeared to be benefits from pre-exposure of fruits to the EOs for 5 days prior to inoculation with *B. cinerea*. Seven days post-inoculation, fungal lesion measurement showed a significant suppression of fungal development by all the examined concentrations of EO in pre-exposed fruits suggesting induced resistance effects (Figure 3.10).

Fungal sporulation measurements in cherry tomato fruits were performed 14 post-inoculation. Interestingly, fungal sporulation decreased with the presence of the EOs when compared to the controls. Spore production was inhibited even with the application of 50 ppm while increasing EO concentration led to greater effects. Examining the viability of the produced spores revealed that only the application of 250 ppm significantly reduced the germination of spores among treatments.

In fruits pre-exposed to volatiles for 5 days before inoculation, ANOVA revealed spore production of *B. cinerea* was significantly reduced in the 250 ppm treatment. However, no effects were observed in spore viability of the produced spores (Table 3.4).



**Figure 3.10:** Impacts of dittany EO volatiles (A) sustained-direct effect and (B) induced resistance effect applications on cherry tomatoes lesion area (cm<sup>2</sup>) of grey mould (*B. cinerea*), 7 days post-inoculation. Values represent mean ( $\pm$ SE) of measurements made on 24 independent fruits per treatment. Treatments followed by the same letter do not differ significantly at  $P=0.05$  according to Duncan's Range Test.



**Figure 3.11:** Impacts of dittany EO volatiles on *B. cinerea* development in inoculated cherry tomato fruits 7 days post-inoculation.

**Table 3.4:** Impacts of dittany EO volatiles (A) sustained-direct effect and (B) induced-resistance effect application on *B. cinerea* sporulation and spore germination on cherry tomato fruits 14 days post-inoculation. In each column mean values (n=12) followed by the same letter do not differ significantly at P=0.05 according to Duncan's Range Test.

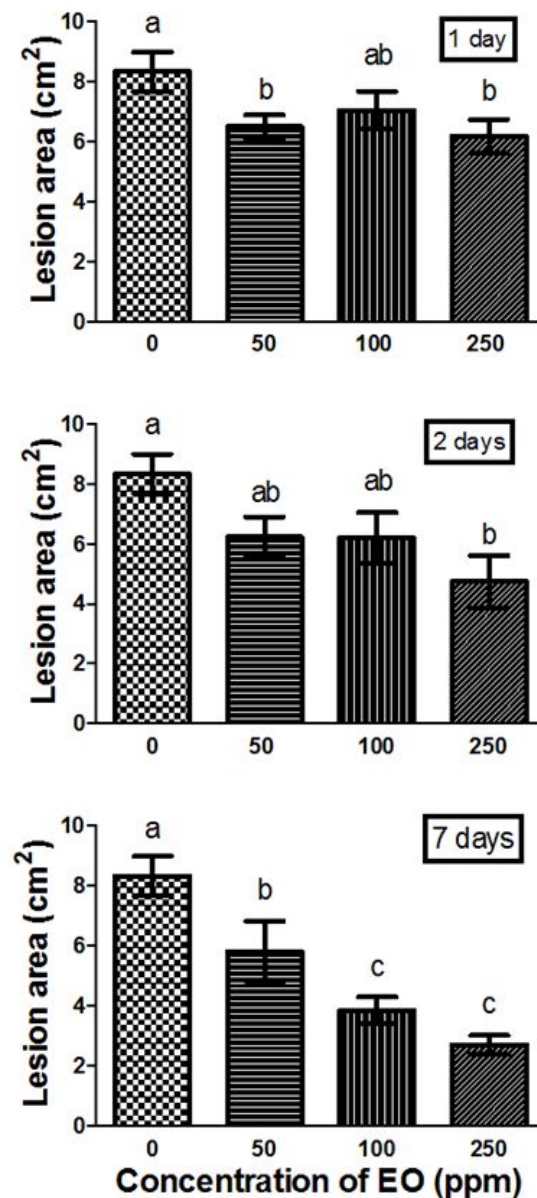
EO (ppm)	(A)		(B)	
	Spore production (10 <sup>5</sup> /mL)	Spore germination (%)	Spore production (10 <sup>5</sup> /mL)	Spore germination (%)
0	85.78 a	99.16 a	130.82 a	99.08 a
50	8.38b	99.12 a	117.03 ab	97.91 a
100	1.72 c	98.58 a	105.03 ab	98.08 a
250	0.14 c	96.33 b	100.50 b	98.41 a

### 3.3.3 Effects of dittany volatiles on *B. cinerea* development in wound-inoculated pepper fruits

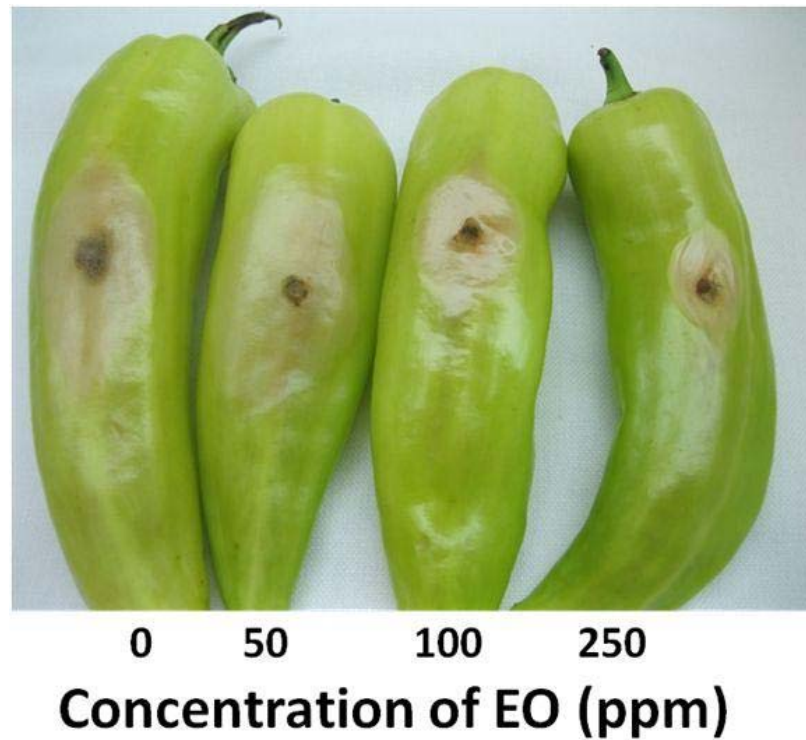
Pepper fruits were inoculated with *B. cinerea* and exposed to dittany volatiles (0, 50, 100, 250 ppm) for 1 or 2 days and continuously (for 7 days before lesion measurements, and after 14 days when spore production was evaluated). In inoculated pepper fruits ANOVA revealed that 7 days of exposure to dittany volatiles suppressed fungal lesion growth even with the lowest application of EO (50 ppm), while higher concentrations resulted in greater effects (see Figures 3.12,3.13). Furthermore, the application of 250 ppm of EO vapours was effective in significantly suppressing *B. cinerea* development in pepper fruits when used for a short time of 1 or 2 days (Figure 3.12).

In fruits pre-exposed to the EOs prior to fungal inoculation, there was an indication of induced resistance to the pathogen, based on the reduced lesion areas. In fruits pre-treated with EO volatiles for a short time (1 or 2 days) the ANOVA revealed that only the 250 ppm treatment for 2 days was effective causing a significant decrease in

lesion size when compared to the untreated controls (Figure 3.14). It should be noted that due to nature of the fruit the pathogen developed in the internal cavity of fruits as well resulting in significant decay and rapid deterioration of the peppers (Plate 3.1).



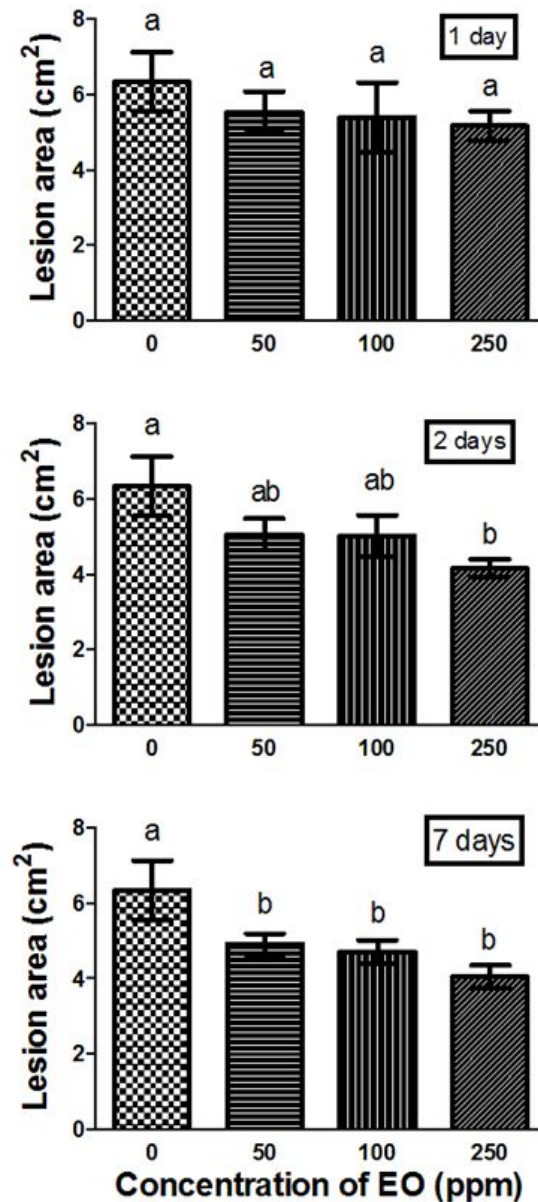
**Figure 3.12** Impacts of concentration (0, 50, 100, 250 ppm) and time of exposure (1, 2, 7 days) of dittany EO volatiles on pepper fruits lesion area (cm²) of grey mould (*B. cinerea*) 7 days post-inoculation. Values represent mean ( $\pm$ SE) of measurements made on six independent fruits per treatment. Treatments followed by the same letter do not differ significantly at  $P=0.05$  according to Duncan's Range Test.



**Figure 3.13:** Impacts of dittany EO volatiles, sustained-direct application (7 days), on *B. cinerea* development on inoculated peppers 7 days post-inoculation.



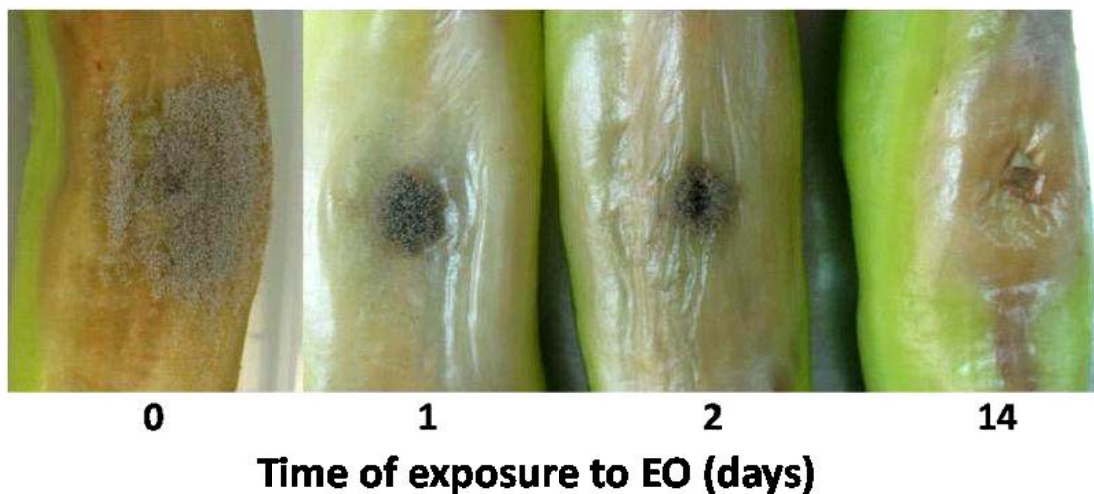
**Plate 3.1:** *B. cinerea* development in internal cavity of pepper fruit 14 days post-inoculation.



**Figure 3.14:** Impacts of concentration (0, 50, 100, 250 ppm) and time of exposure (1, 2, 7 days) of dittany EO volatiles on pepper fruits lesion area (cm<sup>2</sup>) of grey mould (*B. cinerea*) in pre-exposed fruits to volatiles (induced resistance effects), 7 days post-inoculation. Values represent mean ( $\pm$ SE) of measurements made on six independent fruits per treatment. Treatments followed by the same letter do not differ significantly at  $P=0.05$  according to Duncan's Range Test.

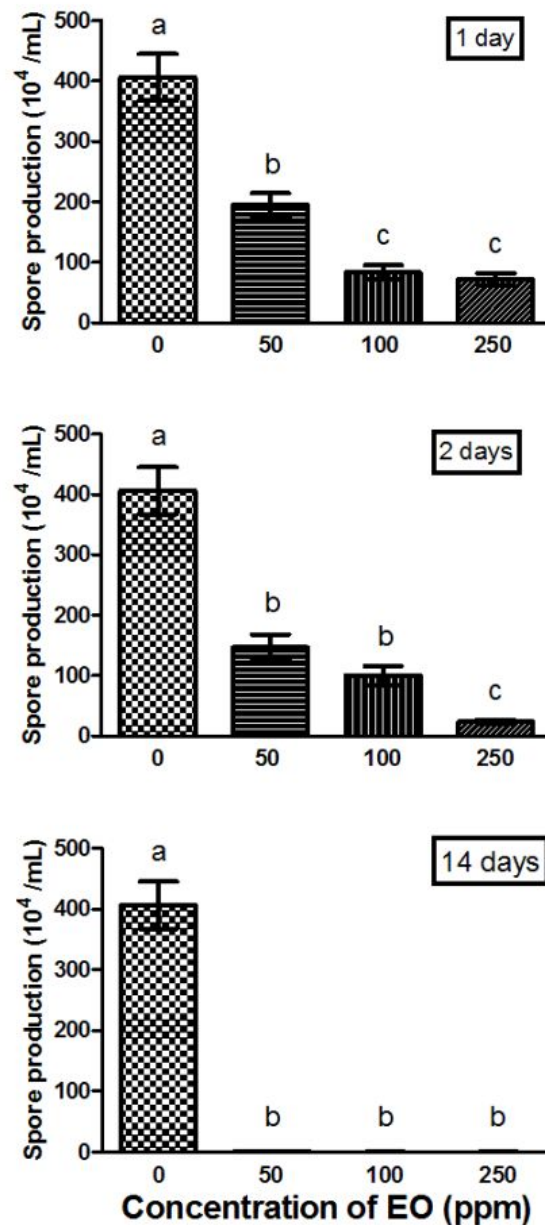


Fourteen days post-inoculation all pepper fruits (EO-treated or controls) showed signs of extensive lesion area. Nevertheless, in EO-treated peppers fungal sporulation was suppressed significantly by all the examined concentrations of EO volatiles and this occurred even after the 1 day EOs treatment (see Figures 3.15, 3.16). In the induced resistance treatments there was a significant reduction in fungal sporulation in the 250 ppm EO after 2 and 14 days exposure (Figure 3.17). However, the viability of the spores produced from the above treatments did not differ (see Figures 3.18, 3.19).

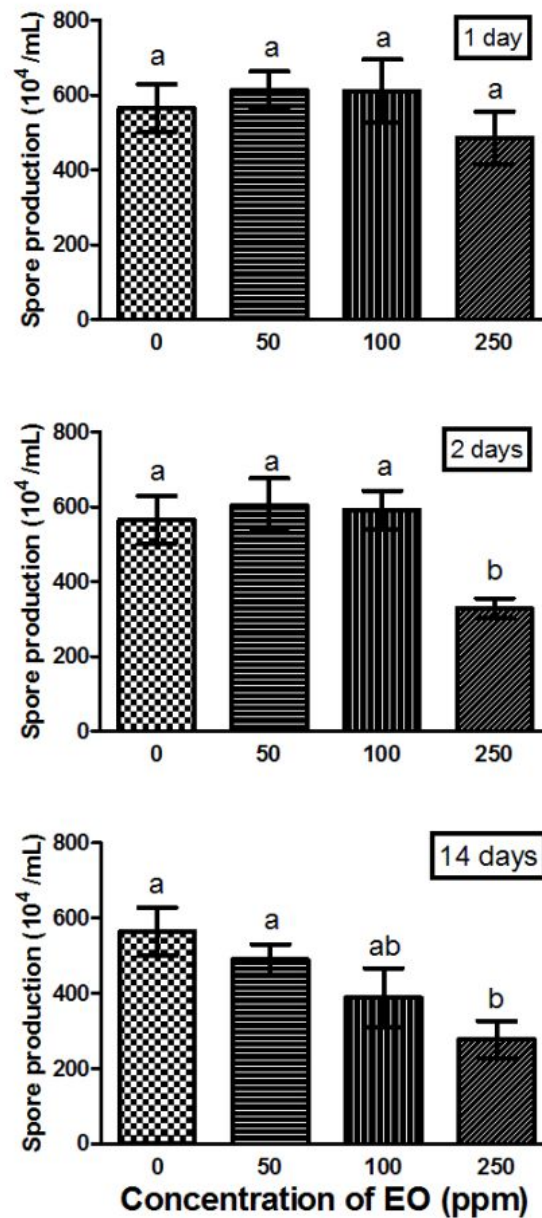


**Figure 3.15:** Effects of the concentration of 250 ppm of dittany EO volatiles on sporulation of *B. cinerea* in inoculated pepper fruits 14 days post-inoculation.

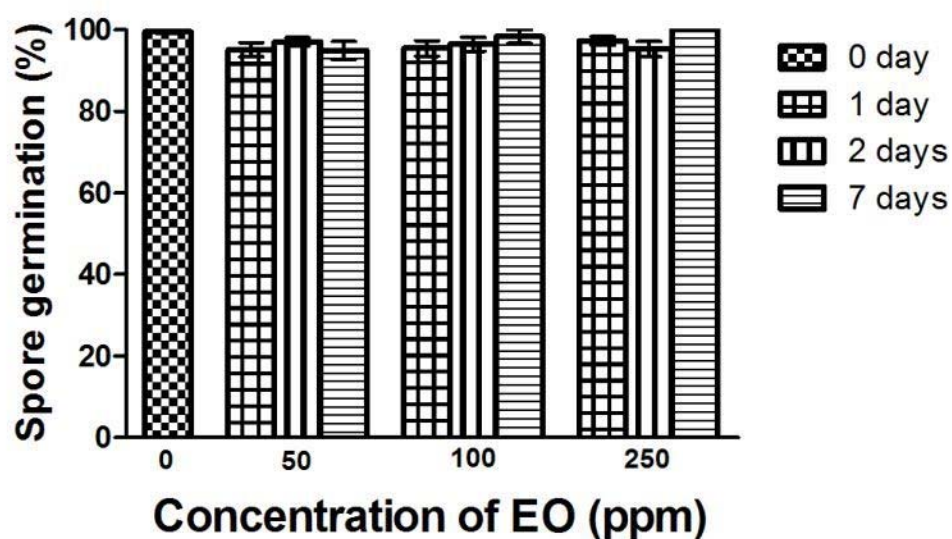




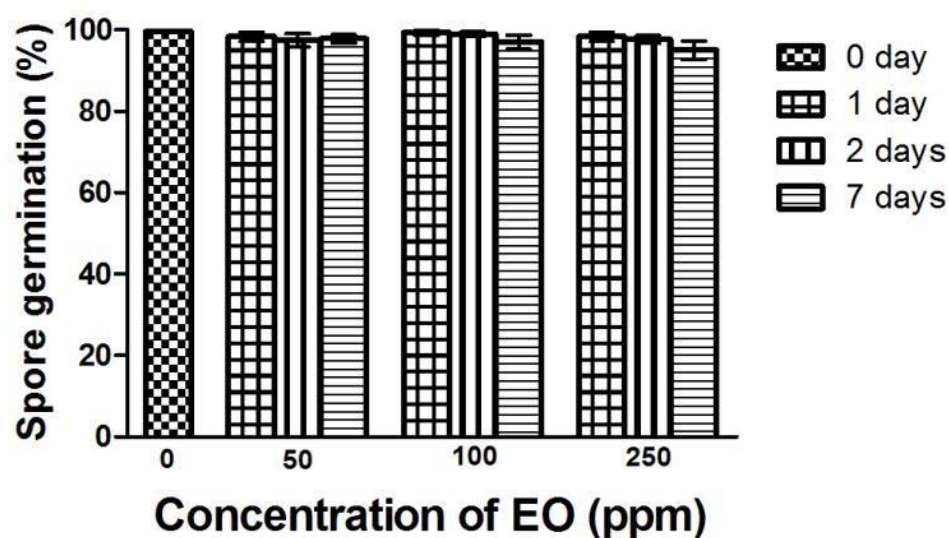
**Figure 3.16:** Impacts of concentration (0, 50, 100, 250 ppm) and time of exposure (1, 2, 7 days) of dittany EO volatiles on *B. cinerea* sporulation in pepper fruits 14 days post-inoculation. Values represent mean ( $\pm$ SE) of measurements made on six independent fruits per treatment. Treatments followed by the same letter do not differ significantly at  $P=0.05$  according to Duncan's Range Test.



**Figure 3.17:** Impacts of concentration (0, 50, 100, 250 ppm) and time of exposure (1, 2, 7 days) of dittany EO volatiles on *B. cinerea* sporulation in pre-exposed pepper fruits to volatiles 14 days post-inoculation. Values represent mean ( $\pm$ SE) of measurements made on six independent fruits per treatment. Treatments followed by the same letter do not differ significantly at  $P=0.05$  according to Duncan's Range Test.



**Figure 3.18:** Impacts of concentration (0, 50, 100, 250 ppm) and time of exposure (1, 2, 7 days) of dittany EO volatiles on *B. cinerea* spore germination of spores collected 14 days post-inoculation in pepper fruits. Values represent mean ( $\pm$ SE) of measurements made on six independent fruits per treatment.



**Figure 3.19:** Impacts of concentration (0, 50, 100, 250 ppm) and time of exposure (1, 2, 7 days) of dittany EO on *B. cinerea* spore germination of spores collected 14 days post-inoculation in pre-exposed pepper fruits to volatiles. Values represent mean ( $\pm$ SE) of measurements made on six independent fruits per treatment.

The overall effect of the EOs on *B. cinerea* development in pepper fruits is shown in Table 3.5. This shows that with an increasing concentration and duration of exposure to the EOs resulted in more effective suppression of grey mould development in pepper fruits.

**Table 3.5:** Impacts of concentration (0, 50, 100, 250 ppm) and time of exposure (1, 2, 7 or 14 days) of dittany EO volatiles on pepper fruits lesion area (cm<sup>2</sup>) of grey mould (*B. cinerea*) 7 days post-inoculation and on pathogen's sporulation 14 days post-inoculation in pepper fruits: (A) inoculated fruits exposed to volatiles and (B) fruits pre-exposed to volatiles then inoculated - induced resistance effects. In each column mean values (n=6) followed by the same letter do not differ significantly at P=0.05 according to Duncan's Range Test.

EO (ppm)	Time of exposure to EO (days)	A		B	
		Lesion area (cm <sup>2</sup> )	Spore production (10 <sup>5</sup> /mL)	Lesion area (cm <sup>2</sup> )	Spore production (10 <sup>5</sup> /mL)
0	0	8.33 a	40.63 a	6.34 a	56.43 ab
50	1	6.50 abc	19.47 b	5.52 ab	61.39 a
50	2	6.23 abc	14.70 bc	5.05 ab	60.42 a
50	7	5.81bcd	0.14 f	4.91 ab	49.01 abc
100	1	7.05 ab	8.35 d	5.39 ab	61.13 a
100	2	6.20 abc	10.05 cd	5.01 ab	59.11 a
100	7	3.85 de	0.03 f	4.70 ab	38.82 bcd
250	1	6.19 abc	7.18 de	5.16 ab	48.55 abc
250	2	4.73 cd	2.37 ef	4.16 b	32.86 cd
250	7	2.70 e	0.03 f	4.05 b	27.70 d

### 3.3.4 Overview of impacts of dittany volatiles on *B. cinerea* development in wound-inoculated fruits

The EOs volatiles obtained from Cretan Dittany plants were found to be effective in suppressing *B. cinerea* development in wound-inoculated eggplant, cherry tomato and pepper fruits in this study. The best control was achieved with the highest concentrations examined. Previous studies report that strawberries (*Fragaria ananassa* Duch.) treated with EO (0.1 mL/L) or tea-tree oil (*Melaleuca alternifolia* L.) reduced the severity of decay during storage at 10°C by 34% when compared to the control (Chanjirakul et al., 2007). Also, Cassia (*Acacia farnesiana* L.) oil at 0.5 mL/L alone or in combination with MgSO<sub>4</sub> (0.25–3% W/V) reduced the percentage of decayed tomatoes (Feng and Zheng, 2007). Furthermore, severity of decay in tomato fruits decreased during storage when treated with cinnamon or eucalyptus oil (0.05–0.5 mL/L) vapours and transfer to chilled air (Tzortzakis, 2007a).

In the present study the dittany EOs vapour applications for 5 days before fungal inoculation seems to induce resistance of fruits against the pathogen by reducing lesion size produced by the pathogen. Previously, pre-exposing tomato fruit to 0.5 mL/L cinnamon vapours for 3 days and then inoculation with fungi, reduced *B. cinerea* and *C. coccodes* lesion development, extending ‘induced resistance effects’ (Tzortzakis, 2009a). However, further investigation of potential essential oil responses to defence-related mechanisms of fruits is needed.

The findings in this study indicate that the application of dittany volatiles in most cases suppressed fungal sporulation in wound-inoculated fruits. The mode of action of volatiles on fungal spores is poorly understood. However, decreased spore germination/production would suppress the spread of the fungus and hence its

capacity for spore production, making natural products of great importance as a postharvest sanitizers in the storage atmosphere and on surfaces (Stavropoulou et al., 2014).

### 3.4 Effect of *O. dictamnus* essential oil volatiles on quality- related attributes of fruits

Eggplant, cherry tomato and pepper fruits treated with dittany volatiles in this study displayed no visible symptoms of injury or other abnormalities even at the highest EO concentration employed and the longest duration of exposure to these volatiles. Additionally, non-inoculated fruits were treated with EOs concentrations (0, 50, 100, 250 ppm) for 7 days and the impacts of volatiles on quality characteristics (colour value measurements, weight loss, total soluble solids-TSS, titratable acidity-TA, pH, and respiration rates) of fruits are shown below.

Eggplant fruit quality was unaffected by exposure to the dittany volatiles (Tables 3.6, 3.7, Figure 3.20).

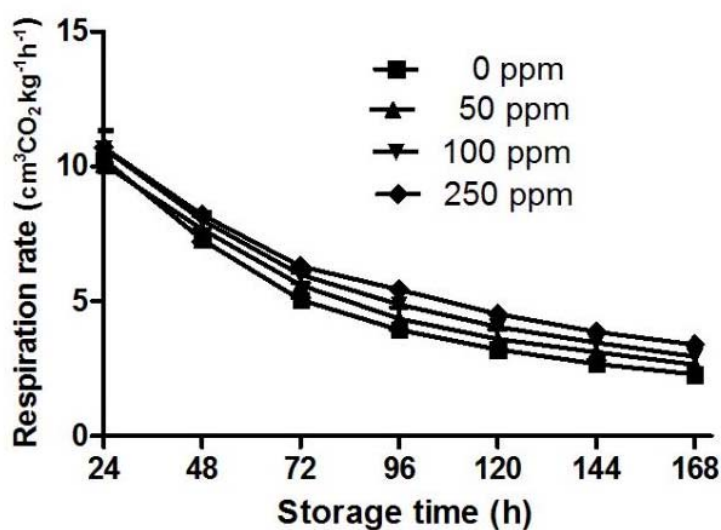
**Table 3.6:** Effects of dittany EO volatiles (0, 50, 100, 250 ppm) on eggplant quality-related parameters following 7 days of vapour enrichment. In each column mean values (n=9) followed by the same letter do not differ significantly at P=0.05 according to Duncan's Range Test.

EO (ppm)	Weight loss (%)	Firmness (Kg cm <sup>-2</sup> )	TSS (°Brix)	TA (% citric acid)	pH
0	1.06 a	13.52 a	4.01 a	0.77 a	5.63 a
50	1.07 a	13.34 a	3.88 a	0.76 a	5.50 a
100	0.97 ab	14.61 a	3.95 a	0.77a	5.54 a
250	0.88b	15.59 a	3.70 a	0.75 a	5.56 a

The application of 50 or 100 ppm of EO increased fruit skin lightness (*L*) compared to the untreated fruits. ANOVA revealed increased pulp lightness in EO-treated fruits compared to the control samples, which would be a positive result as good pulp quality is an important quality criterion for eggplants (Concellón et al., 2007). Fruit weight loss did not change after EO application changes of <1.07 %. No significant differences were observed in total soluble solids, titratable acidity and pH of fruit juice as well as in fruit firmness or calyx colour values and respiration rates of EO-treated fruits when compared to untreated controls.

**Table 3.7:** Effects of dittany EO volatiles (0, 50, 100, 250 ppm) on eggplant colour (calyx, skin, pulp) parameters following 7 days of vapour enrichment. In each column mean values (n=9) followed by the same letter do not differ significantly at P=0.05 according to Duncan's Range Test.

EO (ppm)	Colour						
	Calyx			Skin			Pulp
	L	C	H	L	C	H	L
0	54.88 a	29.87 a	113.08 a	23.47 b	3.73 a	355.68 bc	82.74 c
50	54.72 a	30.03 a	114.56 a	24.22 a	4.63 a	357.27 a	84.44 bc
100	54.52 a	29.72 a	114.25 a	24.68 a	4.21 a	355.39 c	85.10 ab
250	54.07 a	31.21 a	114.60 a	24.08 ab	4.48 a	357.00 ab	86.60 a



**Figure 3.20:** Impacts of dittany EO volatiles (0, 50, 100, 250 ppm) on respiration rates of eggplant fruits. Values represent mean ( $\pm$ SE) of 3 measurements made on 3 independent containers per treatment.

Exposure of cherry tomatoes to dittany volatiles resulted in no significant differences in fruit quality characteristics among the treatments (Tables 3.8, 3.9, Figure 3.21). Similarly, EO applications did not affect pepper fruit quality (Tables 3.10, 3.11, Figure 3.22).

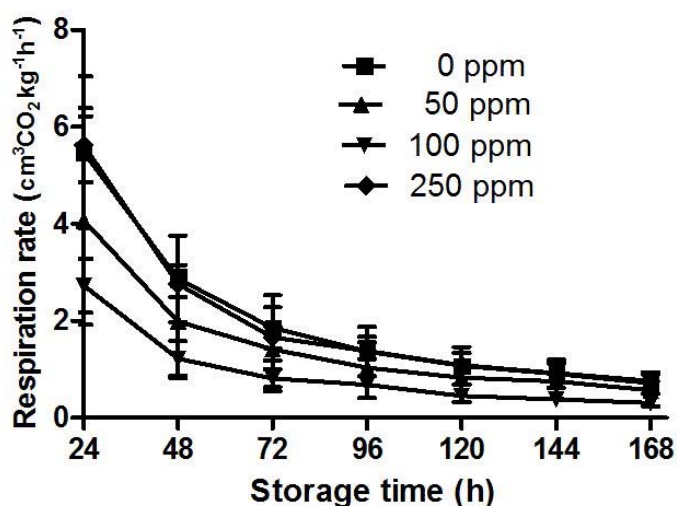
**Table 3.8:** Effects of dittany EO volatiles (0, 50, 100, 250 ppm) on cherry tomato fruits quality-related parameters following 7 days of vapour enrichment. In each column mean values ( $n=12$ ) followed by the same letter do not differ significantly at  $P=0.05$  according to Duncan's Range Test.

EO (ppm)	Weight loss (%)	Firmness (Kg cm <sup>-2</sup> )	TSS (°Brix)	TA (% citric acid)	pH
0	0.48 a	9.55 a	4.36 a	0.416 a	4.096 a
50	0.47 a	9.55 a	4.23 a	0.407 a	4.093 a
100	0.52 a	9.29 a	4.30 a	0.401 a	4.093 a
250	0.50 a	9.51 a	4.36 a	0.418 a	4.106 a



**Table 3.9:** Effects of dittany EO volatiles (0, 50, 100, 250 ppm) on cherry tomato skin colour parameters following 7 days of vapour enrichment. In each column mean values (n=12) followed by the same letter do not differ significantly at P=0.05 according to Duncan's Range Test.

EO (ppm)	Skin colour		
	L	C	H
0	37.52 a	27.95 a	51.88 a
50	37.23 a	27.31 a	51.96 a
100	37.59 a	26.97 a	52.48 a
250	37.51 a	26.94 a	53.40 a



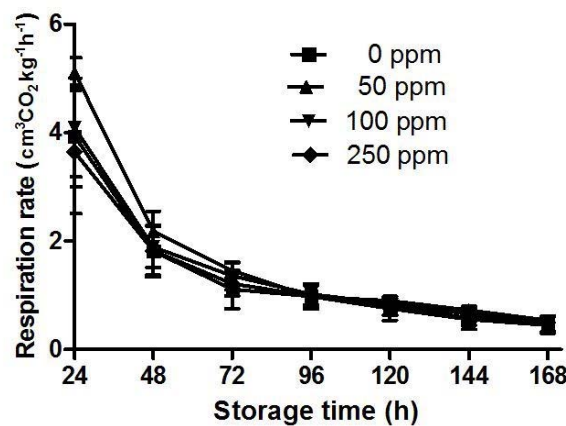
**Figure 3.21:** Impacts of dittany EO volatiles (0, 50, 100, 250 ppm) on respiration rates of cherry tomato fruits. Values represent mean ( $\pm$ SE) of 3 measurements made on 3 independent containers per treatment.

**Table 3.10:** Effects of dittany EO volatiles (0, 50, 100, 250 ppm) on pepper fruits quality-related parameters following 7 days of vapour enrichment. In each column mean values (n=12) followed by the same letter do not differ significantly at P=0.05 according to Duncan's Range Test.

EO (ppm)	Weight loss (%)	Firmness (Kg cm <sup>-2</sup> )	TSS (°Brix)	TA (% citric acid)	pH
0	0.82 a	5.67 a	4.3.1 a	0.073 a	5.64a
50	0.90 a	6.17 a	4.23 a	0.075 a	5.56 a
100	0.79 a	6.53 a	4.16 a	0.195 a	5.60 a
250	0.89 a	5.73 a	4.15 a	0.073 a	5.56 a

**Table 3.11:** Effects of dittany EO volatiles (0, 50, 100, 250 ppm) on pepper fruits skin colour parameters following 7 days of vapour enrichment. In each column mean values (n=12) followed by the same letter do not differ significantly at P=0.05 according to Duncan's Range Test.

EO (ppm)	Skin colour		
	L	C	H
0	55.26 a	33.54 a	111.51 a
50	55.29 a	35.98 a	112.07 a
100	54.95 a	34.67 a	112.05 a
250	54.98 a	35.08 a	111.58 a



**Figure 3.22:** Impacts of dittany EO volatiles (0, 50, 100, 250 ppm) on respiration rates of pepper fruits. Values represent mean ( $\pm$ SE) of 3 measurements made on 3 independent containers per treatment.

Overall, fruits quality-related attributes examined in this study were not affected by the presence of dittany essential oils (up to 250 ppm) while in the case of eggplants some effects on fruit quality were observed. However, further investigation is necessary for the possible changes of the biochemical mechanisms involved. Previous studies reported that antioxidative parameters (i.e., ascorbic acid, total phenols, and lycopene) increased following *origanum* essential oils application in tomato fruits (Tzortzakis et al., 2011) and acetaldehyde vapour-treated avocado delayed fruit softening (Pesis et al., 1998), indicating an induced resistance role of essential oils on the fruits.

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**CHAPTER 4**  
**Conclusions**

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## 4.1 Conclusions

In this study, the effects of dittany (*O. dictamnus* L.) essential oil (EO) volatiles on postharvest grey mould (*B. cinerea*) disease development in eggplant, cherry tomato and pepper fruits were evaluated. Results indicate that with the volatile applications, especially at the highest concentrations of EO employed in this project, pathogen development was inhibited significantly both *in vitro* and in fruits by reduced lesion growth as well as suppressed fungal sporulation. In addition, fruit quality-related parameters were found to be unaffected by the presence of these volatiles. The main results suggest the following:

- Fungal development *in vitro* was completely inhibited by the continued application of 100 or 250 ppm of EO volatiles while inhibitory effects were also marked by lower EO concentration and short time of exposure.
- In inoculated eggplant, cherry tomato and pepper fruits the 50 ppm of EO application resulted in suppressed disease development by reduced lesion growth and fungal sporulation, where increasing EO concentration led to greater effects.
- Exposure of inoculated fruits to EO volatiles for a short time, even for 1 day, resulted in significantly reduced fungal sporulation, clearly shown in the pepper experiment.
- Pre-exposure of fruits to volatiles before fungal inoculation revealed reduced lesion growth, indicating that dittany EO probably caused induced resistance of fruits against the pathogen.

- EO-treated fruits displayed no visible symptoms of injury or other abnormalities even at the highest EO concentration employed and the longest duration of exposure to EO volatiles.
- EO application did not affect quality-related characteristics of fruits while skin lightness and pulp lightness of eggplant fruits was improved under the presence of dittany volatiles.

The findings of the current study support the potential use of dittany EO as a postharvest preservative to control economically important fungal pathogens. Its utilisation can provide good antimicrobial protection against grey mould disease by suppressing fungal development and spread of the disease and in addition can maintain quality of fresh produce. Findings have considerable commercial significance, although each commodity needs to be individually assessed, and the volatile concentration and sanitising technique optimised, before the volatile treatment may be used commercially. Essential oils might not be as effective or broad spectrum as chemical sanitizing agents but their effectiveness is promising and can be improved in conjunction with other alternative postharvest disease control methods.

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